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12	2	2	, Norman	(omit)
12	2	9	380 mμ	390 mμ
62	2	15	0.2 ml.	0.25 ml.
63	(in Fig. 1)		O. D. 260 mμ	O. D. 260 mμ × 10^{-3}
64	(in Fig. 2)		O. D. 260 mμ	O. D. 260 mμ × 10^{-3}
68	2	21	(vetaining	(retaining
98	1 (in Table II)	10 (in Table II)	2/1	1/2
100	1	23, 32, 35, 40, 42, 48	Nagase	Nagarse
136	1	15	*Midium*	*Medium*
138	1	18	comonent	component
138	2	5	comonent	component
141	1	6	extented	extended
142	2 (in Table III)	8 (in Table III)	40	20
147	1	11	dependent	dependent I^{131}
147	1	20 (from bottom)	resuls	results
147	1	2 (from bottom)	alinear	a linear
147	2	legend 4) of Table II	Standard	Standard 33 mili
148	1	17 (from bottom)	does-response	Dose-response
215	1 (in Fig. 7)		open circles	solid circles
248	2	3 (legend of Fig. 3)	preparasion	preparation
251		3 (legend of Fig. 5)	pursfied	purified
253	1 (in Table VII)	1 (in Table VII)	ond	and
259	2	upper pattern (in Fig. 2)		(add Val between Tyr and Ileu)
259		A (in Fig. 3)	DNP-NH_3	DNP-NH_2
260	1	1	(one)	(none)
260	1	3 (foot note)	Pro-Lys-Ale-OH	Pro-Lys-Ala-OH
262	1	5 (foot note)	Pro-LyslOH	Pro-Lys-OH
317～318	2 1	2 (from bottom)～ 9 (from bottom)	On the other hand,～ B_6 compounds.	(omit)
328	1	2 (legend of Fig. 2)	of vitamin B_6	in the absence of vitamin B_6
328	1	7 (from bottom)	preformed	performed
352	2	14 (from bottom)	$C_{28}H_{50}$ C 72.06, H 10.80	$C_{27}H_{48}O_5$ C 71.64, H 10.69
354	1	4 (from bottom)	4-C^{14}-pentahydroxy-coprostane	4-C^{14}-pentahydroxybufostane
458		6 (in Title)	*Phygiological*	*Physiological*
//	2	11	debri	debris
//	//	14	coditions	conditions
//	//	23	silkgrand	silkgland
//	//	33	FDNB-,	FDNB,
459		3 (in Fig. 1)	Silkgrands	Silkglands
//		1 (legend of Fig. 1)	microsomal	microsomal fraction
//	1	2	*Bombix mori* L,,	*Bombyx mori L.*,
//	//	6	Bioreseach Inc., U.S.A	Bioresearch Inc., U.S.A.,
//	2	1, 3	silkgrands	silkglands

Page	Column	Line	For	Read
459	2	8	*Fraction*	*Fractionation*
459	2	13	volums	volumes
//	//	19	buflered	buffered
460	1	3	shaken	shaked
461	1 (in Table II)	11 (in Table II)	and ATP	and ATP-
//	1	4	Tabel	Table
//	//	17	absense	absence
//	2	20	*biz.*	*viz.*
462		1 (legend of Table III)	8 μmoles, $MgCl_2$	8 μmoles $MgCl_2$
//	2	4	following	succeeding
//	//	6	unnessary	unnecessary
463	1	11	confim	confirm
//	//	11	radio	radio-
//	2	1	acid	acids
//	//	8	other	ether
464	(Fraction II in Fig. 3)		0-time 23 c. p. m.	0-time 0 c. p. m.
//	//	6	as	as well as
465		2 (legend of Table VI)	silkgrands	silkglands
//	1	6	it suggested	it is suggested
//	//	25	and C^{14}-	and from C^{14}-
477	3 (in Table I)	6 (in Table I)	62	72
500	1	19	peroxidases 556	peroxidase 556
503	2	6	succes-sively.	successively.
505	2	8	397,	397 mμ
507	2	1	hehavior	behavior
507	2	2	hehavior	behavior
515	1	5	1,000 r. p. m.	10,000 r. p. m.

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609		4 (in Title)	Rosche	Roche
610	1	14 (from bottom)	Calciumlacetat	Calciumlactat
611	1	8	Tokophrol	Tokopherol
611	2	20 (from bottom)	Essigsäu rean-	Essigsäurean-
611	2	14–15 (from bottom)	zwis-chen	zwisc-hen
612	1	1	(add)	Maximums bei *ca.* 325 mμ Anwesenheit von
612	1	23 (from bottom)	Maximums bei *ca.* 325 mμ Anwesenheit von	(omit)
612	2	4 (legend of Fig. 1)	getölet	getötet
613	1	5 (legend of Fig. 3)	Starke	Stärke
620	1	6	borohydrate	borohydride
704	(Fig. 5)		A B	B A

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Chemical Modification of Tuberculin Protein

III. Oxidation and Reduction

By Yoshimi Okada

(*From the Department of Biochemistry, Faculty of Medicine, Kyushu University, Fukuoka*)

(Received for publication, November 25, 1960)

In the previous papers (*1*, *2*) the author reported the chemical modifications of tuberculin active protein, named hC, with 2,4-dinitrofluorobenzene, *p*-phenylazobenzoylchloride and aromatic diazonium compounds. It was suggested in the first paper (*1*), that at least tyrosine residues and half of the lysine residues are not directly concerned in the tuberculin activity. In the second paper (*2*), it was reported that tuberculin activity of sulfanilic acid azo hC, in which about a half of histidine residues was diazotized, was found to be lower than the original hC, and as a possible hypothesis, histidine residues may be concerned in the activity.

The present investigation was undertaken to disclose the importance of histidine residues in hC for the activity by decomposing the residues by means of photooxidation in the presence of methylene blue (*3*, *6*). The modification with photooxidation was often used in enzymatic studies to prove the histidine residues as the essential groups for the activity (*6*).

In addition, the role of disulfide bonds in hC was investigated by oxidizing and reducing them with performic acid and with thiol compounds respectively.

The results obtained in the present investigation seem to suggest that histidine residues are essential and cystine and cysteine residues are not essential for the tuberculin activity.

MATERIALS

Tuberculin Protein—The tuberculin protein, named hC, was prepared from the culture filtrate of human strain Aoyama B according to the method described by Seibert with slight modifications (*1*).

Five hundredth micrograms of hC is as potent as 0.1 ml. of old Tuberculin (×2000 dilution) as reported in the previous paper (*1*, *2*).

METHODS AND RESULTS

Biological Test—Each 0.1 ml. of the solution to be tested and standard tuberculin (0.5 μg. hC per ml. of physiological saline solution) were injected intracutaneously into the same person simultaneously. Skin reaction was measured at 48 hours after the injection.

Reduction of hC with Thiol Compounds—(1) Experiments with cysteine—Solutions, buffered with 0.1 *M* phosphate at pH 7.8, containing 0.02% hC together with a 20-fold amount of cysteine were showed no appreciable loss of tuberculin activity, even when kept for 5 days at room temperature (Table I).

(2) Experiment with thioglycollic acid—Thioglycollic acid was neutralized with 5 *N* sodium hydroxide. Solutions, buffered with 0.1 *M* phosphate at pH 7.8, containing 0.02% hC together with 20-fold amount of thioglycollic acid showed also no appreciable loss of tuberculin activity after standing for 5 days at room temperature (Table I).

Performic Oxidation of hC—(1) Preparation of oxidized hC with performic acid—Performic acid oxidation was carried out according to the method described by Schram *et al.* (*7*) with slight modifications. The performic acid reagent was prepared by the addition of 1 volume of 30% H_2O_2 to 9 volumes of 90% formic acid. The solution was allowed to stand for one hour at room temperature.

The hC was dissolved in formic acid (35 mg. in 1 ml.) and 3 ml. portions were added to 30 ml. of performic acid solution. The oxidation was allowed to proceed on refrigerator overnight. At the end of the reaction time 30 ml. of water and 120 ml. of 20% trichloroacetic acid solution were added with continuous stirring and the resulting precipitate was collected, washed with 10% trichloroacetic acid solution and dissolved in alkaline solution (pH 8.5). The solution was treated again with trichloroacetic acid solution and the precipitate finally formed was washed with ethanol and ether. The product thus obtained and designated as PFO-hC was a white powder. The yield was about 95 mg.

Table I

Tuberculin Activity of Modified hC

Modified hC	Dose (μg.)	Number tested	Average dimension of reaction (mm.)	
			Modified hC	hC
Treatment with cysteine	0.05	10	17.0	18.5
Treatment with thioglycollic acid	0.05	11	19.5	19.5
PFO-hC[1)]	0.05	15	22.5	22.0
PHO-hC (a)[2)]	0.05	5	10.2	31.2
PHO-hC (b)[3)]	0.05	8	10.9	30.4

1) Performic acid oxidized hC.

2) Photooxidized hC. Tuberculin activity was measured in the solution diluted to a concentration corresponding to 0.5 μg. per ml. directly from the reaction mixture containing methylene blue.

3) Photooxidized hC. Tuberculin activity was measured on the solution containing 0.5 μg. of repeatedly precipitated PHO-hC per ml.

(2) Cysteic acid contents of PFO-hC—In order to quantitatively estimate the extent of oxidation of hC with performic acid, cysteic acid content of PFO-hC was determined as follows. PFO-hC was hydrolyzed with 6 *N* HCl in a sealed glass tube for 24 hours at 110°C. After removal of the excess of HCl *in vacuo*, the residual hydrolysate was taken up in a definite volume of 0.1 *M* citrate buffer at pH 3.4. The hydrolysate corresponding to about 10 mg. of original protein was chromatographed on a 0.9×15 cm. column of Dowex-50X8. A 0.1 *M* citrate buffer at pH 3.4 was the eluting solvent. Cysteic acid emerged at the column volume (*8*) and was estimated in the effluent fractions by the photometric ninhydrin technique (*9*). The content of cysteic acid in PFO-hC was 3.2 residues per 10^5 g. of protein.

On the other hand, cystine plus cysteine contents of hC were determined according to the method described by Schram *et al* (*7*). The half cystine content of hC was 3.4 residues per 10^5 g. of protein.

The results indicated that the oxidation of hC by performic acid under the conditions employed proceeded qunatitatively. Toennies (*10*) oxidized casein with performic acid to convert methionine into a sulphone and observed the disappearance of all of the tryptophan. Therefore, although the determination of tryptophan and methionine contents of hC was not made in this report, it seems also very likely that tryptophan and methionine residues in hC was oxidized with performic acid.

(3) Tuberculin activity of PFO-hC—As seen in Table I, PFO-hC was as potent as unmodified hC. This fact suggests that the cystine and/or cysteine, and also probably tryptophan and methionine, in hC are not directly concerned in the activity.

Photooxidation of hC in the Presenc of Methylene Blue—(1) Photooxidation procedure—The manometric technique described by Weil *et al.* (*3*) was used. Ten mg. hC dissolved in 1 ml. of 0.1 *M* phosphate buffer (pH 7.8) was placed in the main chamber of the Warburg vessel. In the side arm of the Warburg vessel was placed 0.2 ml. of a solution containing 0.05% methylene blue, and in the center well of the vessel was placed 0.2 ml. of potassium hydroxide solution. After temperature was equilibrated at 37°C, the dye solution was tipped into the main chamber and the illumination started. The light was supplied by

200-W white light lamp placed at a distance of 20 cm. from the Warburg vessels.

As seen in Fig. 1, the reaction practically came to a standstill after 300 minutes of illumination. The oxygen uptake was amounted to 95 μl. From this data it was calculated that 43 moles of oxygen were taken up into 10^5 g. of hC.

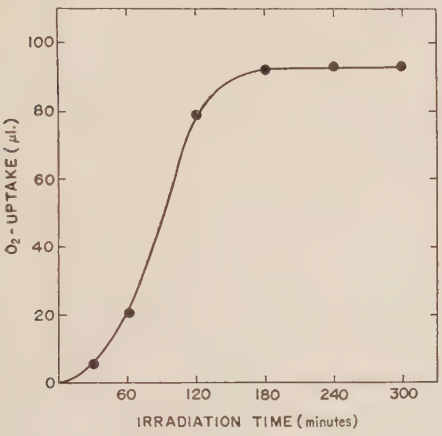

Fig. 1. Photochemical action of methylene blue on hC.

A part of the solution was diluted with physiological saline solution to a concentration corresponding to 0.5 μg. of original protein per ml., and used for the measurement of the tuberculin activity. As seen in Table I (PHO-hC (a)) it was demonstrated that tuberculin activity of the photooxidized hC in the presence of methylene blue was inactivated markedly. Control experiment carried out on the hC, which was irradiated without the addition of methylene blue, showed no appreciable loss of tuberculin activity.

(2) Preparation of photooxidized hC (PHO-hC)—Photooxidized hC was prepared from the solution above mentioned as follows. The dye was removed with a small amount of charcoal. To the filtrate a equal volume of 20% trichloroacetic acid solution was added with continuous stirring and the resulting precipitate was collected, washed with 10% trichloroacetic acid solution and dissolved in 0.01 N NaOH. The solution was treated again with trichloroacetic acid solution. This procedure was repeated twice and the precipitate finally formed was washed with alcohol and ether. The product was designated as PHO-hC.

The physiological saline solution containing 0.5 μg. PHO-hC per ml. was prepared and used for the testing of tuberculin activity. As seen in Table I (PHO-hC (b)) the tuberculin activity of the PHO-hC was markedly lower than the original hC.

(3) Histidine contents of PHO-hC—Previous works of Weil *et al.* (*4*, *5*) have demonstrated that aromatic amino acids, especially histidine and tryptophan, are highly susceptible to the photochemical action of methylene blue. Particular attention therefore, was paid to histidine. Tryptophan was not determined because, as can be seen from the result of performic acid oxidation of hC, it was assumed that tryptophan was not essential for the tuberculin activity.

Histidine was determined according to the method of Moore and Stein (*11*). The elution curve was presented in Fig. 2. No

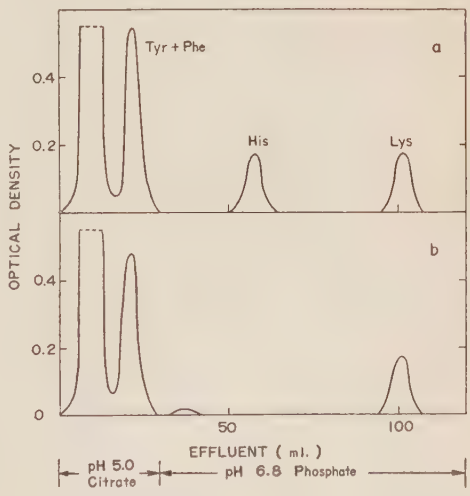

Fig. 2. Histidine analysis of hC and PHO-hC using 0.9×15 cm. column of Dowex-50X8. (a) hC. (b) PHO-hC

histidine was recovered from the hydrolysate of PHO-hC. It was demonstrated, therefore, that all histidine residues in hC were oxidized

by photochemical action of methylene blue and it was also suggested that histidine residues are concerned in the activity.

DISCUSSION

The reduction with thiol compounds and/or the oxidation with performic acid of hC had no effect on the tuberculin activity. It is clear that disulfide bonds and/or sulfhydryl groups in hC are not directly concerned in the tuberculin activity.

Furthermore, it has demonstrated that a secondary structural alteration in the hC molecule following the cleavage of disulfide bonds had no effect on the tuberculin activity. It would be seen, generally, that one of the roles of the disulfide bonds is to provide an additional stability to the secondary structure, and the rupture of the disulfide bonds leads to a more unfolding of the molecule, and to an inactivation as can be seen in example of trypsin (*12*) and ribonuclease (*13*). In the case of hC, with the cleavage of disulfide bonds of hC with thiol compounds and/or with performic acid, in contrast, no inactivation of original activity can be detected. Since hC was prepared from the culture filtrate sterilized by heating at 100°C for 30 minutes, it is more probable that hC is denaturated protein and contains no three-dimensional regular orientation in molecule. In addition, no inactivation occurred even after the hC solution was heated at 100°C for one hour at pH 7 or 9.*

From these data it was concluded that the nature of the secondary structure of hC molecule was not essential for the tuberculin activity.

Tryptophan and methionine residures in protein can also be oxidized with performic acid (*10*, *14*). Although the determination of the contents of these amino acids was not performed on PFO-hC, it seems very likely that tryptophan and methionine residues of hC are not directly concerned in the activity.

It was shown that, when hC was photo-oxidized by visible light in the presence of traces of methylene blue, inactivation of hC took place along with the decomposition of all of the histidine residues in the molecule. From this data, it is certain that histidine residues are closely concerned in the activity. The view will be supported by the results of the previous paper (*2*), demonstrating the loss of the tuberculin activity of hC by diazotization with sulfanilic acid diazonium compound.

In the chemical study of rabbit anti-ovalbumin, Porter (*15*) reported that rupture of the disulfide linkage by reduction with thioglycollate had little effect on the combining powder of the antibody, and it seems probable that the combining site of the antibody is rather restricted on a particular group or groups in the molecule. As above mentioned, breaking of the disulfide bonds or heat denaturation of hC had no effect on tuberculin activity. Further it was demonstrated in the previous paper that amino and phenolic groups are not essential for the activity (*1*). It seems, therefore, very probable that the active site of hC restricted on a particular group and independent of secondary structure of the molecule. These results seem to suggest that the active unit of tuberculin can be in a relatively low molecular state and tuberculin active peptide may be prepared from the partial degradation product of tuberculin protein. In this connection it is interesting that the highly potent tuberculin active peptide was isolated from the bodies of tubercle bacilli (*16*, *17*).

Table II, together with the results of the previous papers (*1*, *2*), lists the summary of the effect of reagents on hC and tuberculin activity. It was suggested that only histidine residues, at least among the residues modified by the author, was essential for the activity.

SUMMARY

1. Tuberculin active protein, hC, was treated with cysteine and thioglycollic acid but the tuberculin activity was not affected.

2. The hC was oxidized with performic acid. Tuberculin activity remained unchanged even after all of the cystine and/or cysteine

* Okada, Y., unpublished data.

TABLE II

Effect of Reagents on hC and Tuberculin Activity

Reagents	Amino	Imidazole	Indole	Disulfide	Sulfhydryl	Phenol	Tuberculin activity
Dinitrofluorobenzene	##	+		−	##	##	no loss
Phenylazobenzoylchloride	##			−		+	no loss
Aminophenol azo compound	−	+		−		+	no loss
Sulfanilic acid azo compound	+	##		−		##	decrease
Thiol compound	−	−	−	##	−	−	no loss
Performic acid	−	−	##	##	##	−	no loss
Photooxidation	−	##	##			+	decrease

The symbols used have the following significance; ##, #, and + indicate the relative extent of reaction, denoting the most extent of reaction with ##. Minus indicates the negative reaction.

residues were oxidized to cysteic acid.

3. When hC was photooxidized by visible light in the presence of methylene blue, inactivation of the tuberculin activity of hC took place along with the decomposition of all of the histidine residues in the molecule.

4. It is probable that histidine residues are essential, but cystine or cysteine residues are not essential for the activity.

The author wished to thank Prof. Akabori, Osaka University, for his continuous interest and advice during the course of this investigation, and to thank Prof. Yamamura, Kyushu University, for his encouragement and guidance.

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Regeneration of Amino Acid and Peptide from their Trinitrophenyl Derivatives under the Action of Ammonia*

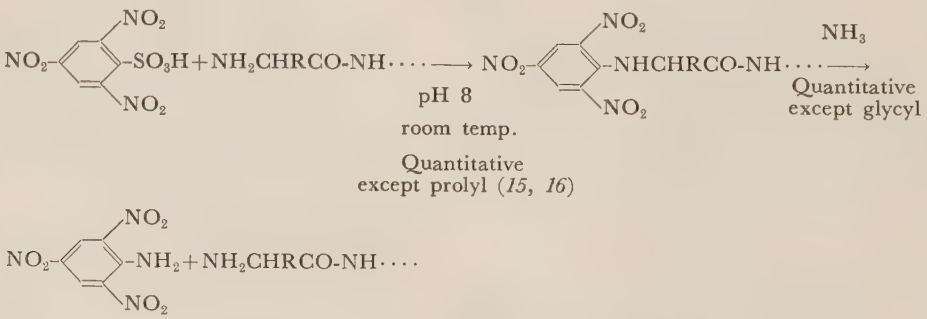
By Kazuo Satake, Masaru Tanaka and Haruo Shino

(From the Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Tokyo)

(Received for publication, December 15, 1960)

Dinitrophenylamino acids derived from the N-terminals of peptide or protein with the use of 1-fluoro-2,4-dinitrobenzene method (*1–4*) have been identified by chromatography on paper (*2–5*) or on columns of Celite (*2, 6*), silisic acid (*1, 2, 7*), ion exchange resine (*2, 8*), polyamide (*9, 10*) or chlorinated rubber (*11*). In special circumstances, their identities have necessiated to be further verified by heating dinitrophenylamino acid with ammonia (*12*), barium hydroxide (*13*) or hydroiodic acid (*14*) and paper chromatographic analyses of the regenerated amino acid. Such a regeneration, however, has been achieved only with unsatisfactory recovery and, in some cases, together with undesirable side-reactions giving another ninhydrin-positive substances (*12–14*).

Recent studies on the TNBS technique of Satake (*15–19*) indicated that TNP-amino acid or -peptide could be ammonolyzed into picramide and the original amino acid or peptide, with excellent yield and without any undesirable side-reactions. These results are described in this paper.



EXPERIMENTALS

Materials—TNP-Amino acids and -peptides used were the same preparations as described in the previous paper (*15, 16*).

Authentic picramide was prepared from picrylchloride with ammonium carbonate in hydrated ethanol (*15, 20*) and was crystallized from ethyl acetate. Fine yellow needles, m.p. 186–188°C (decomposition).

Methods—TNP-Compound to be de-TNP-ated, was dissolved into ammonia at a concentration of one to three μmoles per ml., and the orange-colored solution was kept at room temperature for a given period or was heated in a sealed tube at 110°C for ten minutes. Otherwise mentioned, the concentration of ammonia was 15 *N* (d: 0.90).

The resulting solution was analyzed both by spectrophotometry (after being acidified with large amount of ethanolic hydrogen chloride or being extracted with ethyl acetate) and by paper chromatography. For the quantitative determination of amino acid or peptide regenerated, use was made of 'internal marker method' (*4, 21*).

Thus the reaction mixture was concentrated to dryness *in vacuo*, and the residues were dissolved into distilled water containing a definite amount of an amino acid (leucine or alanine) which had been previously proved to be absent in the original residues. An aliquot of the solution was used as the sample for paper chromatographic resolution, and each spot of

* The following abbreviations are used in this paper; TNP for 2,4,6-trinitrophenyl and TNBS for 2,4,6-trinitrobenzene 1-sulfonic acid.

amino acid or peptide which was stained by spraying acidic ninhydrin reagent and heating the paper at 60°C for five minutes (*22*), was directly densitometried at 570 mμ (485 mμ in the case of proline). From the relative ratio between the areas surrounded by base line and color intensity curve of the product to be assayed (a) and of the amino acid added as the internal marker (b), the amount of amino acid or peptide present in the original residues (x) was calculated according to the following formula:

$$\frac{x}{y}=\frac{a}{b}\quad\frac{Ca}{Cb}$$

where Ca and Cb represented the 'leucine equivalent' of the product and of the amino acid added, under these conditions, respectively. Experiments indicated the accuracy to be ± 5 per cent*.

To avoid photo-decomposition of TNP-compounds (*15*, *16*), the out-side of vessels used through these procedures, was covered with aluminum foil.

RESULTS

TNP-Amino acid did an irreversible change with a moderate velocity under the action of ammonia at room temperature, but did scarcely under the action of sodium hydroxide at the same pH and at the same temperature**. Fig. 1 shows the ultraviolet absorption spectra of mixture in which TNP-glutamic acid (30 mg.) and 15 *N* ammonia (140 ml.) had been preincubated at room temperature for a given period and then acidified with large amount of alcoholic hydrogen chloride, together with those of authentic TNP-glutamic acid and picramide. The result suggested that TNP-NH group in TNP-glutamic acid might be splitted off as picramide, with a velocity according to the following formula:

$$C=C_0e^{-kt}$$
$$K=1/\tau=80\,\mathrm{min}^{-1}$$

where C_0 and C represent the concentrations of TNP-glutamic acid at the initial stage (t=0) and after t minutes, respectively (see Fig. 2).

Such a presumption on the reaction of

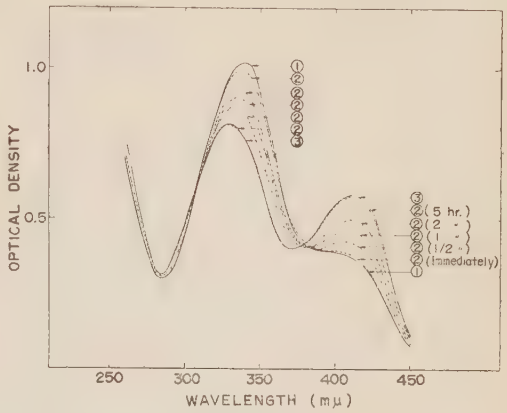


Fig. 1. Absorption spectra of TNP-glutamic acid and picramide.

1: TNP-Glutamic acid

2: TNP-Glutamic acid after preincubation with 15 *N* ammonia at room temperature for a given period

3: Picramide

Solvent: Hydrated alcohol (containing HCl)

Concentration: 0.625×10^{-4} mole per liter.

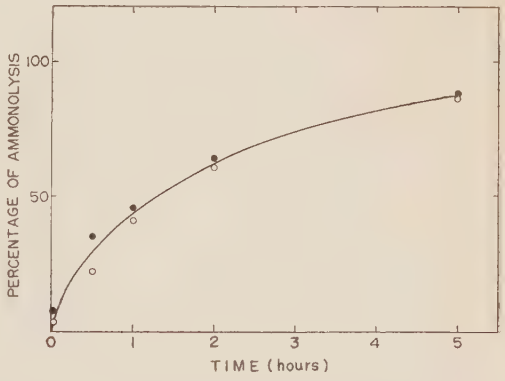


Fig. 2. Ammonolysis of TNP-glutamic acid. The rate of conversion was calculated from the increase (—○—) or the decrease (—●—) of the optical densities at 410mμ or at 340 mμ, respectively (See Fig. 1).

The solid line expresses the rate calculated from $C=C_0e^{-kt}$ and $k=80\,\mathrm{min}^{-1}$. The reaction was performed in 15 *N* ammonia at 20°C.

TNP-glutamic acid with ammonia, was verified by the actual isolation of picramide from the reaction mixture after 5 hours. On ex-

* We were greatly indebted to Miss U. Inoue for her assistance in this analysis.

** It was reported by Hirayama (*23*) that TNP-alanine was obtained with good yield by TNP-ating alanine with picrylchloride in the presence of *N*-sodium hydroxide at room temperature (see also (*15*)).

traction with ethyl acetate, most part of the orange-colored substance(s) was removed from ammonia alkaline layer, different from the intact TNP-glutamic acid which should be remained in aqueous layer under these conditions. Evaporation of the combined ester extracts gave fine yellow crystals, m.p. 186–188°C (decomposition) which showed no depression on admixture with authentic picramide (m.p. 186–188°C) λ_{max} at 327 mμ and 413 mμ (in ethyl acetate). The optical density of ester-extract indicated almost quantitative conversion of TNP-glutamic acid into picramide.

Fig. 3 summarises the paper chromatograms of the reaction mixture at various stages. With the liberation of picramide, these also produced free glutamic acid. Densitometric determination indicated that

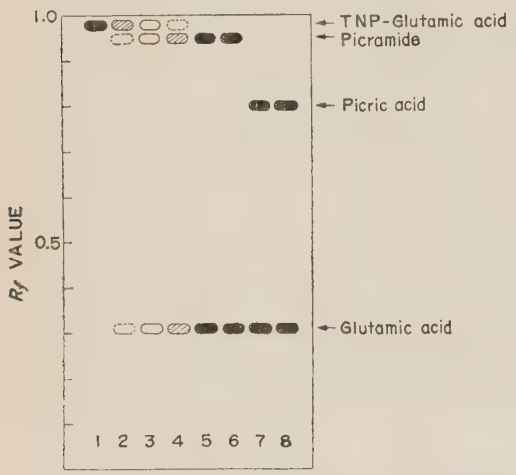


Fig. 3. Paper chromatograms of reaction mixture of TNP-glutamic acid with ammonia or sodium hydroxide.

Sample: Reacted with 15 N NH_3 at room temperature for (1) 0, (2) 1/2, (3) 1, (4) 2, and (5) 5 hours. Reacted with 15 N NH_3 at 110°C for (6) 10 minutes and (7) one hour. (8) Reacted with N $Ba(OH)_2$ at 100°C for 10 minutes.

Chromatographed with a mixture of n-butanol, acetic acid and water (4:1:1, v/v).

the latter amounts were approximately equimolecular to those of picramide, at the all stages of reaction (1/2 to 5 hours).

Such an ammonolysis of TNP-NH group was observed not only on TNP-α-amino acids, but also on ω-TNP-amino acids (ε-mono-TNP- and α-ε-bis-TNP-lysines) and TNP-peptides, producing picramide and the corresponding de-TNP-compounds without any hydrolysis of amide and peptide linkages. Only exceptions were TNP-proline which was fairly labile in ammonia at room temperature, and TNP-glycine and -glycyl peptide which produced appreciable amount of unidentified colored product(s) and only slight amount of glycine and picramide (compare Fig. 4 with Fig. 1). In general, the higher the concentration of ammonia, the greater the velocity of ammonolysis*, but the relation was fairly different from one TNP-compound

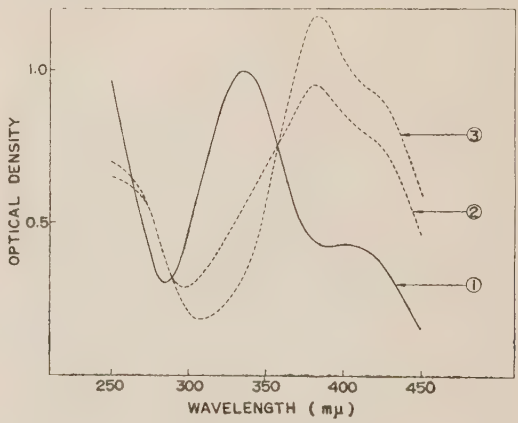


Fig. 4. Absorption spectra of reaction mixture of TNP-glycylglycine with ammonia.

1: TNP-Glycylglycine

2: TNP-Glycylglycine+NH_3 (immediately after mixing)

3: ,, (after 1/2 to 2 hours)

to other. Thus TNP-leucine, -valine, -phenylalanine, -alanine, -methionine and -leucyltyrosine did quantitative conversion, while TNP-tyrosine, -asparagine and -alanylasparagine 80 per cent, and TNP-aspartic acid, -threonine and -serine 60 % conversions, under the action of 15 N ammonia at room temperature for 20 hours, respectively.

On elevating the reaction temperature,

* TNP-Glutamic acid and -valine were ammonolyzed with ammonia diluted as low as $N/2$.

the ammonolysis was greatly accelerated without accompanying any undesirable side-reactions. Under the action of 15 *N* ammonia at 110°C, ammonolysis of TNP-amino acid and -peptide including TNP-prolyl, was completed within only 5 to 10 minutes and with almost quantitative yield. TNP-glycine and -glycyl peptide were exceptions in this case, too. Thus the main product was unidentified compound(s) colored orange, and the yields of picramide and de-TNP-ated product were as low as 35 to 55 per cent. These results are summarized in Table I.

Table I

Recoveries of Amino Acids and Peptides from the Corresponding TNP-Derivatives

TNP-	Yield[1] (%)
Glycine	33[2]
Alanine	97
Valine	102
Leucine	100
Serine	103
Threonine	99
Aspartic acid	101
Glutamic acid	102
Lysine (α-, ε-bis)	100
Arginine	89
Phenylalanine	100
Tyrosine	99
Methionine	99
Cystine (α-, α'-bis)	82
Proline	90
Tryptophane	99
Histidine	105
Asparagine	98
β-Alanine	90
Glycylglycine	55[2]
Alanylasparagine	65
Leucylglycine	90
Leucyltyrosine	93

1) An average error was ±5 per cent.

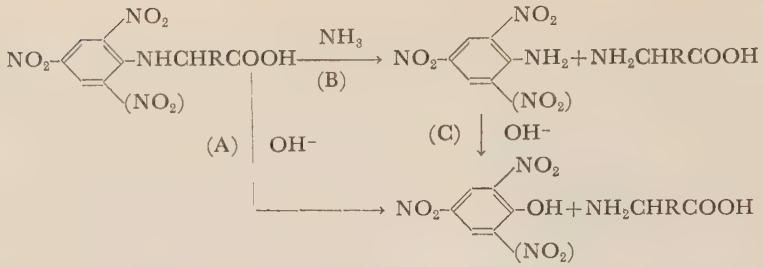
2) The yield of picramide was also as low as 30 to 35 per cent, although there remained no unreacted TNP-glycine.

Discussion

As shown in Table I, TNP-amino acids and -peptides could be de-TNP-ated into the corresponding amino acids and peptides under the action of ammonia, with an excellent yield and without any undesirable side-reactions such as hydrolysis (of amide, peptide and guanidino linkage) or other decompositions (of thioether, disulfide and α-amino-β-hydroxy groups (*12–14*, *24*)). The main product derived from TNP-group was picramide, and the amount was nearly equimolecular to that of de-TNP-ated compound, either the reaction proceeded completely (at 110°C) or incompletely (at room temperature) 1~5 in Fig. 3). The only exception was TNP-glycine and -glycyl peptide which were decomposed partly by ammonolysis (35 to 55 per cent) and partly by other unidentified reaction(s).

On a prolonged reaction at 110°C, there was produced another yellow compound which was presumed to be picric acid from the chromatographic and spectrographic behaviours (See 7 in Fig. 3). Previously Satake (*17*) reported that picric acid was the main product derived from TNP-NH group under the action of ammonia at 100°C. This will be presumably due to the secondary conversion of picramide first produced into picric acid, under the conditions used. These facts seemed to suggest that the similar regeneration of amino acid from the dinitrophenyl derivative with ammonia (*12*), also occured *via* dinitroaniline, which would further change into dinitrophenol without any accumulation. Recently the present author* found that TNP-amino acid was hydrolyzed into picric acid and the corresponding amino acid, under the action of barium hydroxide at 100°C (8 in Fig. 3). The relative ratio of the both reaction velocities, hydrolysis (with *N* barium hydroxide) and ammonolysis (with 15 *N* ammonia), however, was fairly different from one TNP-amino acid to other. As to the ratio of recoveries of amino acid from the dinitrophenyl de-

* Satake, K., Unpublished.



rivative with ammonia and with barium hydroxide, there was reported similar difference (*12*, *13*). It will be another suport that the de-dinitrophenylation with ammonia was also not a simple hydrolysis (A) but initiated by ammonolysis (B) followed with the hydrolysis of dinitroaniline formed (C).

With the only exception of glycyl peptide, TNP-ation followed with the de-TNP-ation, seemed to possess various application in protein chemistry: Thus oligopeptides may be trinitrophenylated, and the resulting TNP-derivatives, after being fractionated by solvent-extraction, talc-column (*15*), chromatography* or counter current distribution*, may be ammonolyzed into the original peptide for analyses by amino peptides, phenylthiocarbamylation and so on. The lysyl linkages in peptide may be reversibly masked with TNP group during tryptic hydrolysis, and such a protection of free amino group with TNP may be also effective for peptide synthesis. There have been used several methods to protect free amino group, but TNP seems to be a sole group which is able to be splitted with alkaline medium. In this connection, it should be also emphasized that the TNP-ating reagent, TNBS, reacts strictly with primary amino group (*15*, *16*), different from the usual acyl, aryl or alkyl reagents.

Summary

1. TNP-Amino acid could be ammonolyzed to picramide and the corresponding amino acid with an excellent yield, under the action of ammonia at 110°C for few minutes.

2. Under these conditions, not only TNP-group in α-amino acid, but also in ω-amino acid or in peptide was similary de-TNP-ated without any undesirable side-reactions, such as hydrolysis or other decompositions.

3. The only exceptions were TNP-glycine and -glycyl peptide, the ammonolysis of which was accompanied with appreciable amount of unidentified side-reaction(s).

4. These results were discussed mainly in connection with protein chemistry.

* Satake, K., Unpublished.

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Stero-Bile Acids and Bile Sterols

XXXVI. Isolation of a New Bile Sterol, 3α, 7α, 12α, 26-Tetrahydroxy-Δ²³-bishomocholene, from Bull Frog Bile

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It has been demonstrated that the C^{24}-bile acid is biosynthesized *via* trihydroxycoprostanic acid from cholesterol (*1–3*). On the other hand, the bile sterols, pentahydroxybufostane (*4*) and tryhydroxyhomocholenes (*5*, *6*), isolated from respective bile of toad and bull frog could be regarded as important intermediates in the conversion of cholesterol to the normal bile acids.

In this paper, the authors report to isolate besides the above sterols (*5*, *6*) a new bile sterol, 3α, 7α, 12α, 26-tetrahydroxy-Δ²³-bishomocholene from bull frog bile by reversed phase partition chromatography.

EXPERIMENTAL AND RESULT

Bull frog bile dissolved in 95% alcohol was refluxed on a water bath for half an hour and then filtered. The filtrate was evaporated to dryness. The residue was dissolved in water, acidified with dilute HCl and extracted with ether to remove acidic substances. Neutral substance in the aqueous layer containing the sterol-sulfate (*5*, *6*) was salted out with NaCl. Twenty grams of this substance were hydrolyzed with 2.5 *N* NaOH by heating in a sealed metal container for 8 hours at 160°C. The hydrolyzate was poured into a large amount of water to separate a bile sterol mixture and then filtered. The bile sterol mixture was then subjected to reversed phase partition chromatography by use of the solvent systems described by Bergström, Norman and Sjövall (*7*). The following phase systems were used.

Phase system	Moving phase	ml.	Stationary phase	ml.
A	Methanol-water	180 : 120	Chloroform-heptane	45 : 5
C	Methanol-water	150 : 150	Chloroform-isooctanol	15 : 15
F	Methanol-water	165 : 135	Chloroform-heptane	45 : 5
M	Isopropanol-water	150 : 150	Chloroform-heptane	15 : 60

Hostalene was used as supporting material for stationary phase. The effluent was collected in test tubes by using an automatic fraction collector. Concentrated H_2SO_4 was added to suitable aliquots of the effluent at room temperature and after an hour extinction at 380 mμ was read to record the concentration of sterol as shown in the following figures. The method of Sjövall (*8*) was used for paper chromatography of bile sterols. Chromatography was carried out at a constant temperature of 23°C.

1) Isolation and Chemical Constitution of the New Sterol

One hundred fifty milligrams of hydrolyzed bile sterol were chromatographed with phase system A and displayed 2 or 3 peaks as shown in Fig. 1.

The effluents of Curve I in Fig. 1 were combined and then the solvent was evaporated to dryness. The residue (50 mg.) was rechromatographed with phase system C and the extinction curve obtained was shown in Fig. 2.

The extinction curve obtained (Fig. 2) was cut into three portions *i. e.*, Curve I_1, Curve I_2 and Curve I_3. The effluents of Curve I_3 were combined and then the solvent

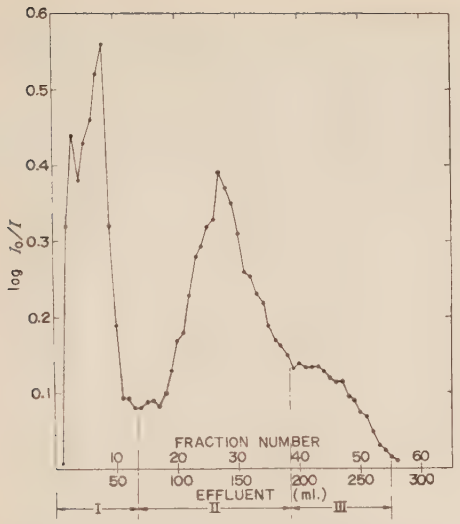


Fig. 1. Column chromatography of hydrolyzed bile sterol of bull frog. Column, 13.5 g. of Hostalene; phase system A.

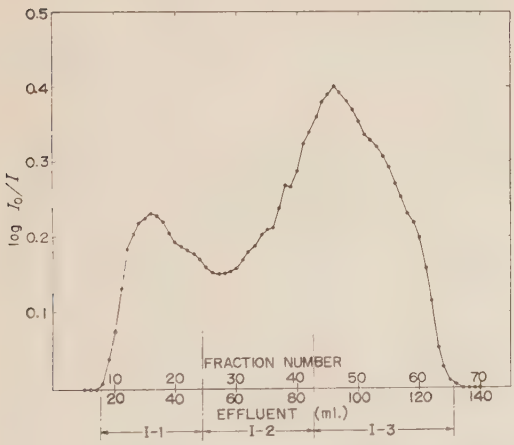


Fig. 2. Rechromatography of Curve I in Fig. 1. Column, 4.5 g. of Hostalene; phase system C.

was evaporated. The residue was rechromatographed with phase system F and gave a curve shown in Fig. 3.

Bile Sterol of m.p. 198°C—The effluents of the curve in Fig. 3. were combined and then the solvent was evaporated. The residue was crystallized from ethyl acetate-methanol to give crystals melting at 198°C. The Hammarsten reaction of this sterol was positive and bromine in glacial acetic acid was de-

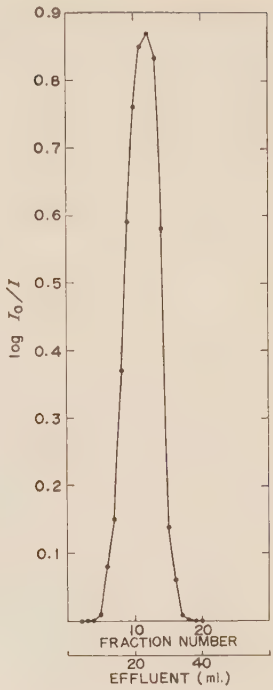


Fig. 3. Rechromatography of Curve I_3 in Fig. 2. Column, 4.5 g. of Hostalene; phase system F.

colored. In the infrared spectrum (Fig. 4) of this sterol, a strong band was found at 963 cm^{-1}. This indicates that this sterol contains a trans double bond at the side chain. Analytical data of this sterol were as follows:

Analysis Calcd. for $C_{26}H_{44}O_4$: C, 74.24, H, 10.54
Found: C, 73.65, H, 10.39

Oxidation of the Sterol; 3, 7, 12-Triketo-Δ^{23}-bishomocholenic Acid—To a solution of 300 mg. of the above sterol in 10 ml. of glacial acetic acid, 300 mg. of chromic anhydride in 0.5 ml. of water and 10 ml. of glacial acetic acid were added drop by drop. After standing at room temperature for an hour, the reaction mixture was diluted with water, and the resulting precipitate was filtered off, washed, dried and crystallized from methanol-water. Crystals were dissolved in an aqueous solution of Na_2CO_3, and then acidified with dilute HCl, and the resulting precipitate was filtered off, washed and dried. Recrystallization of this precipitate from ethyl acetate-methanol gave 150 mg. of crystals with m. p.

225–31°C. The ultra-violet spectrum of this product showed the absence of α, β-unsaturated carboxylic grouping. Infrared spectrum of this acid is shown in Fig. 5.

Analysis Calcd. for $C_{26}H_{36}O_5$: C, 72.86, H, 8.47
Found: C, 72.73, H, 8.52

water, and the solvent was evaporated. Ninety seven mg. of the residue were chromatographed with phase system C (Fig. 6).

The effluents (from 34 ml. to 90 ml.) of the curve in Fig. 6 were combined and then the solvent was evaporated. The residue was

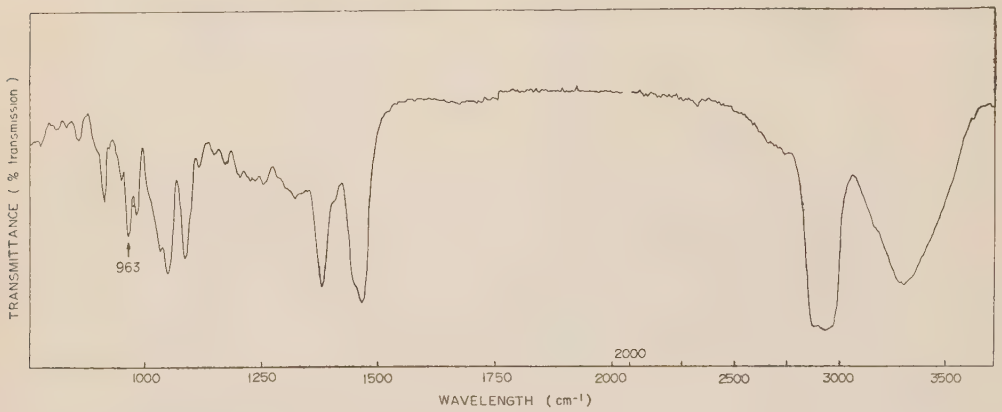


Fig. 4. Infrared spectrum of 3α, 7α, 12α, 26-tetrahydroxy-Δ^{23}-bishomocholene.

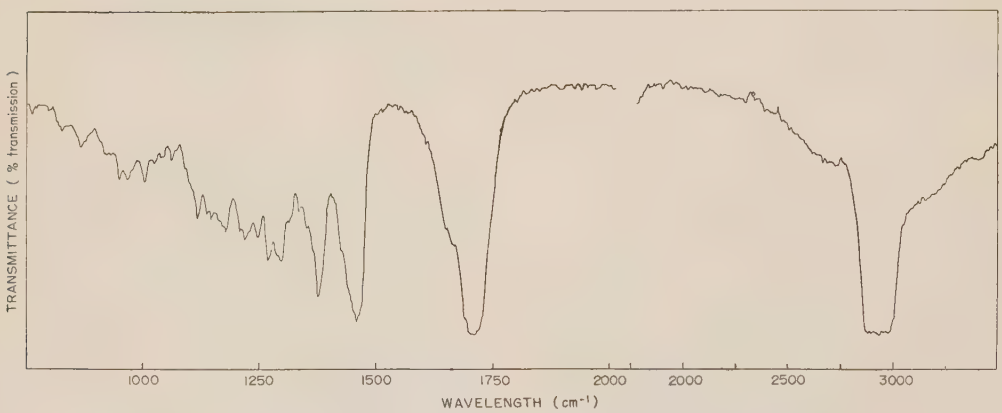


Fig. 5. Infrared spectrum of 3, 7, 12-triketo-Δ^{23}-bishomocholenic acid.

Norcholic Acid—Four hundred milligrams of tetrahydroxy-Δ^{23}-bishomocholene (m. p. 198°C) dissolved in glacial acetic acid was ozonized for 30 minutes and then the solvent were evaporated under reduced pressure. Sixity milliliters. of water were added to the residue and then the solution was hydrolyzed on a water bath for 5 hours. The hydrolyzate was alkalified with Na_2CO_3, and insoluble residue was filtered off. The filtrate was acidified with dilute HCl and extracted with ether. The ether extract was washed with dissolved in water, acidified with dilute HCl, and extracted with ether. The ether extract was washed with water, dried and evaporated. The residue was recrystallized from acetone-water to give crystals with m. p. 189°C. Melting point of this acid was not depressed, when mixed with authentic norcholic acid. Infrared spectra and paper chromatograms (Fig. 11) of the two compounds were also in good agreement with each other.

Analysis Calcd. for $C_{23}H_{38}O_5$: C, 70.01, H, 9.71
Found: C, 69.47, H, 9.67

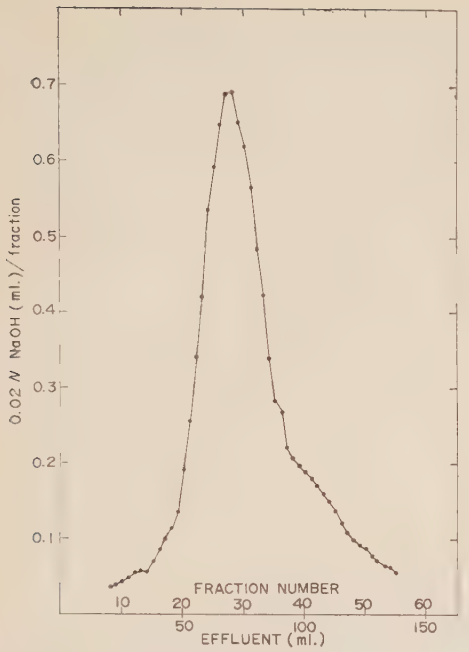


Fig. 6. Column chromatography of the acid obtained from ozonide. Column, 9 g. of Hostalene; phase system C. Solid line, titration curve.

3α, 7α, 12α, 26-Tetrahydroxybishomocholane—Two hundred mg. of tetrahydroxy-Δ^{23}-bishomocholene dissolved in glacial acetic acid were hydrogenated with 20 mg. of platinum oxide catalyst at room temperature. Theoretical volume of hydrogen was absorbed within 5 minutes. After removal of the platinum catalyst, the solvent was evaporated to dryness. Recrystallization of the residue from ethyl acetate-methanol gave crystals with m.p. 200–1°C. Infrared spectrum of these crystals was identical with that of synthesized 3α, 7α, 12α, 26-tetrahydroxybishomocholane (see below). R_f values of paper chromatography (Fig. 12) of both sterols were also identical.

Analysis Calcd. for $C_{26}H_{46}O_4$: C, 73.88, H, 10.97
Found: C, 74.14, H, 11.26.

3, 7, 12-Triketobishomocholanic Acid—To a solution of 100 mg. of tetrahydroxybishomocholane in 5 ml. of glacial acetic acid, 100 mg. of chromic anhydride in 0.3 ml. of water and 3 ml. of glacial acetic acid were added drop by drop. After being allowed to stand at room temperature for an hour, the reaction mixture was diluted with water and the resulting precipitate was dissolved in alkaline solution of Na_2CO_3, and acidified with dilute HCl. The precipitate was recrystallized from methanol to give triketobishomocholanic acid with m.p. 227–8°C. Melting point of this acid was not depressed when mixed with synthesized 3, 7, 12-triketobishomocholanic acid (*9*).

Analysis Calcd. for $C_{26}H_{38}O_5$: C, 72.52, H, 8.90
Found: C, 72.35, H, 8.71.

2) *Isolation of Other Sterols from Bull Frog Bile*

The effluents of Curve II in Fig. 1 were combined and the solvent was evaporated. The residue was recrystallized from methanol-water to give a lot of needles of trihydroxyhomocholene (m.p. 178.5°C), and a small amount of needles of m.p. 238°C which may be an isomer of the former. The chromato-

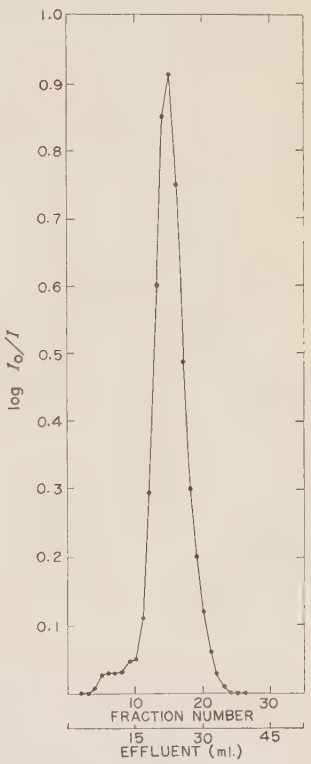


Fig. 7. Column chromatography of trihydroxyhomocholene. Column, 4.5 g. of Hostalene; phase system M.

gram of tryhydroxyhomocholene with phase system M was shown in Fig. 7 (the peak at 22.0 ml.). The chromatogram of the isomer with phase system M was shown in Fig. 8 (the peak at 22.0 ml.). R_f values of paper chromatogram of trihydroxyhomocholene and its isomer were shown in Fig. 13.

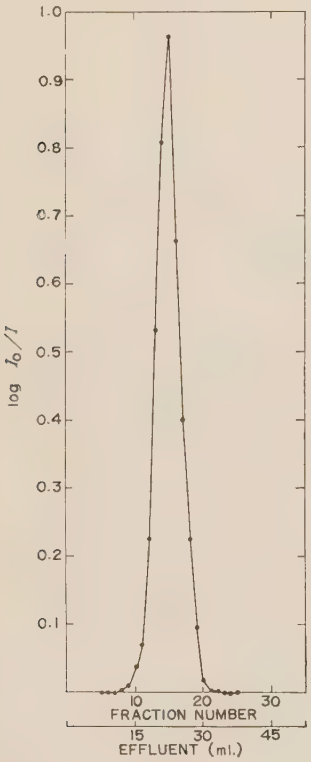


Fig. 8. Column chromatography of isotrihydroxyhomocholene. Column, 4.5 g. of Hostalene; phase system M.

The effluents of Curve III in Fig. 1 were combined and the obtained sterol was recrystallized from methanol-water to give crystals of needle, another new sterol with m. p. 182–3°C. Chemical constitution of this sterol will be published in the future.

The effluents of Curve I_1 in Fig. 2 were combined and the solvent was evaporated. The residue was rechromatographed with phase system C giving a curve as shown in Fig. 9. The effluents from 20 ml. to 50 ml. in this curve were combined and then the solvent was evaporated to dryness. The residue was not crystallized from any of the solvents used. Accordingly, determination of chemical constitution of this new sterol was impossible, but this sterol may probably be a pentahydroxycoprostane judging from the position of the peak on the chromatogram as shown in Fig. 9.

Fig. 10 shows rechromatogram of the

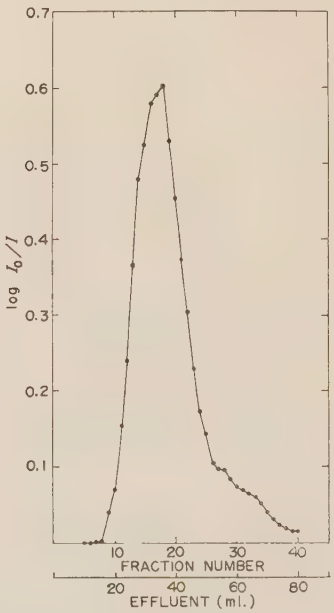


Fig. 9. Rechromatography of Curve I_1 in Fig. 2. Column, 4.5 g. of Hostalene; phase system C.

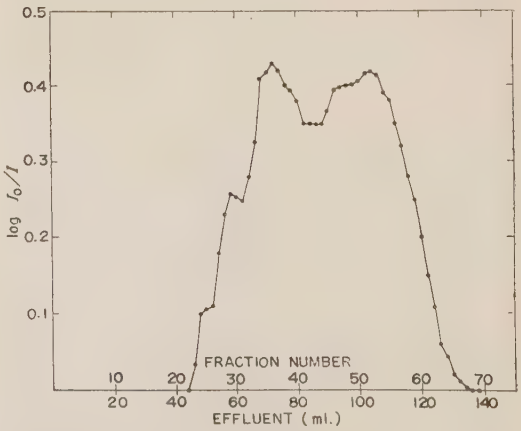


Fig. 10. Rechromatography of Curve I_2 in Fig. 2. Column, 4.5 g. of Hostalene; phase system C.

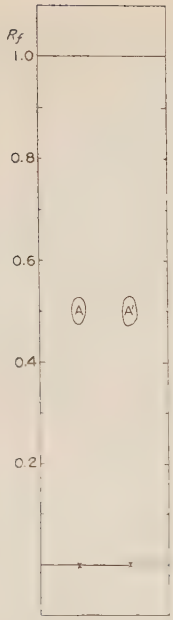


Fig. 11. Paper chromatogram of authentic norcholic acid (A) and the acid (A′) obtained from the ozonide. Mobile phase, ethylene chloride-heptane 60 : 40; stationary phase, 70% acetic acid.

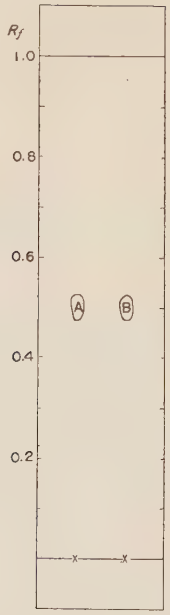


Fig. 13. Paper chromatogram of trihydroxy-homocholene (m.p. 178.5°C) (A) and its isomer (m.p. 238°C) (B). Mobile phase, isopropyl ether-heptane 20 : 80; stationary phase, 70% acetic acid.

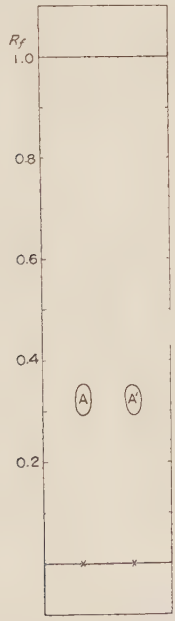


Fig. 12. Paper chromatogram of synthesized 3α, 7α, 12α, 26-tetrahydroxybishomocholane (A) and saturated bile sterol (A′) obtained by hydrogenation of the sterol with m.p. 198°C. Mobile phase, isopropyl ether-heptane 60 : 40; stationary phase, 70%, acetic acid.

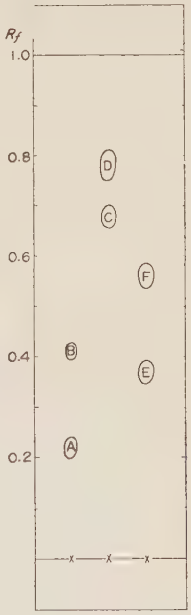


Fig. 14. Paper chromatogram of bile sterols obtained from Curve I_1 (A, B) and Curve I_2 (C, D) in Fig. 2, tetrahydroxybishomocholene (D), pentahydroxybufostane (E), and 3α, 7α, 12α, 24-tetrahydroxycholane (F). Mobile phase, ethylene chloride-heptane 60 : 40; stationary phase, 70% acetic acid.

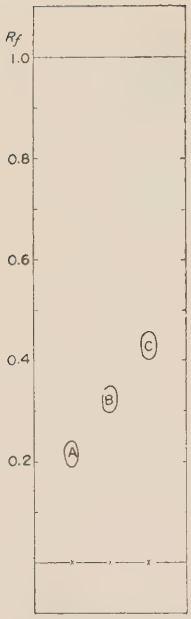


Fig. 15. Paper chromatogram of synthetic bile sterols: 3α, 7α, 12α, 25-tetrahydroxyhomocholane (A), 3α, 7α, 12α, 26-tetrahydroxybishomocholane (B) and 3α, 7α, 12α, 27-tetrahydroxycoprostane (C). Mobile phase, isopropyl ether-heptane 60:40; stationary phase, 70 per cent acetic acid.

sterol obtained from the effluents of Curve I_2 in Fig. 2. This sterol was not crystallized from any of the solvents used. Paper chromatograms of bile sterols obtained from Curve I_1 and I_2 were shown in Fig. 14.

3) *Reference Substances Used for Identification of the Bile Sterols*

3α, 7α, 12α, 26-Tetrahydroxybishomocholane— Methyl bishomocholate was prepared according to the procedure in the previous report in this series (*10*). To a solution of 100 mg. of lithium aluminum hydride in 10 ml. of dry ether, a solution of methyl bishomocholate with m.p. 152° in 10 ml. of dry ether was added under cooling during 10 minutes. After being allowed to stand at room temperature for an hour, the reaction mixture was slowly poured into a mixture of crushed ice and 5 per cent sulfuric acid. Separated ether layer was washed with water dried, and the solvent was evaporated to dryness. The semi-crystalline residue was hydrolyzed with 20 ml. of 5% methanolic KOH for 2 hours on a water bath. The hydrolyzate was diluted with water and the resulting precipitate was filtered off and digested with ether-ethyl acetate. The extract was washed with water, dried and concentrated under reduced pressure. The residue was purified by chromatography on Hostalene column with phase system F, and crystallized from ethyl acetate. Recrystallization from methanol-water gave a pure sample of 3α, 7α, 12α, 26-tetrahydroxybishomocholane (m.p. 202°C).

3α, 7α, 12α, 25-tetrahydroxyhomocholane— Tetrahydroxyhomocholane was prepared by the analogous method as described above from methyl homocholate obtained according to the method of Kazuno *et al.* (*11*). It was purified by chromatography and crystallized from ethyl acetate to give 3α, 7α, 12α, 25-tetrahydroxyhomocholane (m.p. 189°C).

3α, 7α, 12α, 27-Tetrahydroxycoprostane— Methyl trihydroxycoprostanate (*5*) dissolved in dry ether was treated with lithium aluminum hydride as described above. Hydrolysis and chromatography of the reaction mixture gave tetrahydroxycoprostane (m.p. 201°C). Paper chromatograms of these three synthetic tetrahydroxy-sterols are shown in Fig. 15.

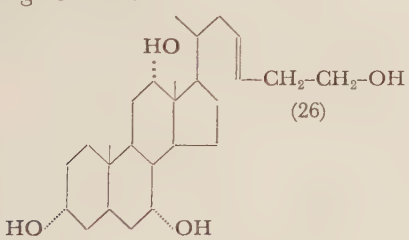
DISCUSSION

Four kinds of bile sterol were isolated by use of reversed phase partition chromatography from bull frog bile. Among these sterols, trihydroxyhomocholene (m.p. 178.5°C) and its isomer (m.p. 238°C) had been isolated by Kazuno (*5*) and Kuroda (*6*). The other new bile sterols melted at 198°C and 182–3°C, respectively. It was also confirmed that besides these sterols there exist some kinds of bile sterols, though in minor amounts, in the bile of bull frog.

One of the new sterols was characterized as 3α, 7α, 12α, 26-tetrahydroxy-Δ^{23}-bishomocholene (m.p. 198°C). It decolored bromine in glacial acetic acid and its infrared spectrum showed the presence of trans ethylenic linkage. Chromic anhydride oxidation of this sterol gave an unsaturated triketo acid melting at

225–31°C. Infrared and ultraviolet spectra of this dehydro-compound showed the presence of carboxyl group, but not of $\alpha\beta$-unsaturated carboxyl group. By these results it was confirmed that this sterol had both double bond and primary alcohol group in the side chain, but was not an $\alpha\beta$-unsaturated alcohol. The localisation of the double bond between C_{23} and C_{24} was clearly demonstrated by the finding that the decomposition of the ozonide of this sterol gave norcholic acid. On the other hand, this sterol easily absorbed one molecule of hydrogen, giving a saturated sterol.

Infrared spectrum and paper chromatogram of this saturated sterol were identical with those of synthesized 3α, 7α, 12α, 26-tetrahydroxybishomocholane. Moreover, melting point of triketobishomocholanic acid obtained by oxidation of the saturated sterol was not depressed when mixed with synthesized 3, 7, 12-triketobishomocholanic acid. From these results, it was concluded that the new bile sterol (m.p. 198°C) was 3α, 7α, 12α, 26-tetrahydroxy-Δ^{23}-bishomocholene as shown in the following formula.



Masui injected cholesterol-4-C^{14} intraperitoneally into bull frog and isolated radioactive 3α, 7α, 12α, 26-tetrahydroxy-Δ^{23}-bishomocholene from its bile (*12*). So it will be an interesting problem to be solved, whether the C_{24}-bile acid is formed by direct oxidation of trihydroxycoprostanic acid (*1–3*) or *via* the C_{26}-intermediates, like the sterol here isolated.

SUMMARY

1. Two new and two already known bile sterols were isolated from bull frog bile by use of reversed phase partition chromatography.

2. It was verified that chemical constitution of one of the new bile sterols (m.p. 198°C) corresponded to 3α, 7α, 12α, 26-tetrahydroxy-Δ^{23}-bishomocholene.

3. 3α, 7α, 12α, 25-Tetrahydroxyhomocholane, 3α, 7α, 12α, 26-tetrahydroxybishomocholane and 3α, 7α, 12α, 27-tetrahydroxycoprostane were synthesized.

The authors wish to thank Prof. S. Bergström of the Karolinska Institute who has kindly given Hostalene.

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Stero-Bile Acids and Bile Sterols

XXXIX. Metabolism of Lithocholic Acid

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(Received for publication, January 9, 1961)

In the early experiment of Bergström *et al.* it was demonstrated that in bile fistula rat, lithocholic acid was converted to unidentified dihydroxycholanic and trihydroxycholanic acids, the latter of which was not identical with cholic acid (*1*). No further report about the identification of these metabolites has appeared so far.

Recent investigations in this laboratory indicated a wide distribution of lithocholic acid in the bile and feces of various animal species (*2—4*), and it seemed of interest in elucidate the biosynthesis and metabolic processes of this bile acid. In this communication, the metabolism of lithocholic acid in the liver of rat and guinea pig is presented.

MATERIALS AND METHODS

Lithocholic [24-C^{14}] acid was prepared by the method of Bergström *et al.* (*5*) and purified by column chromatography and recrystallization to a constant specific activity of 5×10^6 c. p. m./mg. DPN was prepared by the method of Kornberg (*6*), and TPN, by the method of Horecker and Kornberg (*7*). Rat and guinea pig liver homogenates were prepared respectively with 4 parts of the Bucher medium (*8*) per 1 part of liver. Cell debris was centrifuged off at $800\times g$ for 5 minutes in the cold and the supernatant was used as the homogenate. A typical experiment performed was as follows: 38 ml. of 20% liver homogenate was incubated with 2 ml. of phosphate buffer containing 40 mg. of ATP-Na, 4 mg. of DPN, 4 mg. of TPN and 1 mg. of sodium lithocholate [24-C^{14}] dissolved in 0.2 ml. of ethanol. The incubation mixture was shaken in air for 1 hour at 37°C and 5 volumes of ethanol was added to stop the reaction. The mixture was heated for 10 minutes in a boiling water bath, cooled, and the precipitate was filtered off.

The filtrate was evaporated to dryness and the residue was saponified with 2.5 *N* sodium hydroxide by heating in a sealed metal container for 8 hours at 140°C. The hydrolyzate was acidified with dilute hydrochloric acid and extracted twice with ether. The ethereal extract was washed, evaporated to dryness, and subjected to reversed-phase column partition chromatography with phase system F (chloroform: heptane=45:5 as stationary phase and methanol: water=165:135 as moving phase), Hostalene being used as supporting material (*9*). Each 2 ml. portion of the eluate was collected and its radioactivity was determined in an infinite thin layer by a windowless gas-flow counter.

RESULTS

Fig. 1 shows a typical chromatogram obtained when lithocholic acid was incubated with rat liver homogenate. As shown in the figure, lithocholic acid was metabolized into more hydrophilic compounds that left the column earlier. This result is in accord with that of Bergström *et al.* who likewise injected radioactive lithocholic acid into bile fistula rats (*1*). The first band (effluents from 5 ml. to 15 ml.) which behaves similar to a trihydroxycholanic acid on the column was proved to be a mixture of at least three compounds by paper chromatography, but no further identification was attempted at this time, and it will be the subject of future publications.

The labeled material in the effluents from 17 ml. to 31 ml. (the peak at 23.3 ml.) was identified with 3α, 6β-dihydroxycholanic acid by radioautography of the paper chromatogram obtained by using the phase system of 70% acetic acid as stationary phase and isopropyl ether: heptane (60:40) as moving phase (*10*). As shown in Fig. 5, radioactivity

TABLE I

Successive Crystallization of 3α, 6β-Dihydroxycholanic [24-C¹⁴] Acid from Various Solvents

No.	Crystallization solvent	Weight (mg.)	Total activity (c. p. m.)	Specific activity (c. p. m./mg.)
1	ethyl acetate	25.1	7,600	303
2	acetone-water	22.9	7,500	327
3	methanol-water	21.5	7,200	312
4	ethanol-water	19.1	6,270	328

TABLE II

Successive Crystallization of Formylchenodesoxycholic [24-C¹⁴] Acid from Various Solvents

No.	Crystallization solvent	Weight (mg.)	Total activity (c. p. m.)	Specific activity (c. p. m./mg.)
1	ethanol	79.5	385,000	3,880
2	ethanol-water	45.7	200,000	4,380
3	acetone-water	26.1	95,000	3,660
4	ethyl acetate	20.4	86,200	4,220

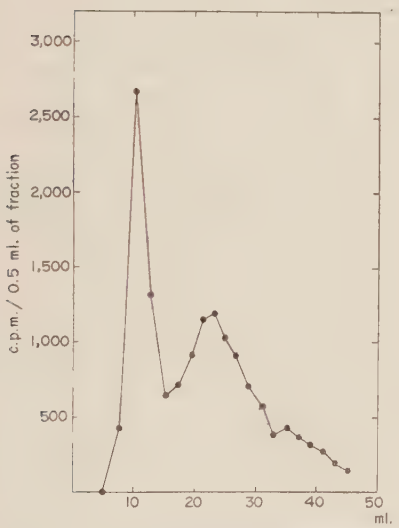

FIG. 1. Partition chromatography of the metabolites of lithocholic acid incubated with rat liver homogenate. Column: 4.5 g. of Hostalene, phase system F (chloroform: heptane=45:5 as stationary phase and methanol: water=165:135 as moving phase)

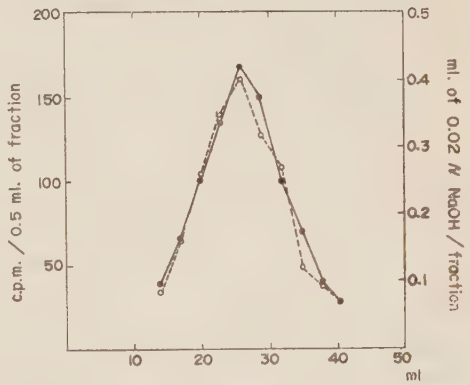

FIG. 2. Rechromatography of the labeled material obtained from the effluents from 17 ml. to 31 ml. shown in Fig. 1 after cocrystallization with unlabeled 3α, 6β-dihydroxycholanic acid. Column: 4.5 g. of Hostalene, phase system F (chloroform/heptane=45:5 as stationary phase and methanol/water=165:135 as moving phase
—— Radio-activity Curve, ... Titration Curve

was found in an area corresponding to the same position as authentic 3α, 6β-dihydroxycholanic acid. Additional confirmation was obtained by cocrystallization to a constant specific activity with unlabeled 3α, 6β-dihydroxycholanic acid (Table I) and by a subsequent rechromatography of the recrystallized material (Fig. 2).

The labeled material in the third band (the effluents from 35 ml. to 40 ml.) was identified with chenodeoxycholic acid by radioauto-

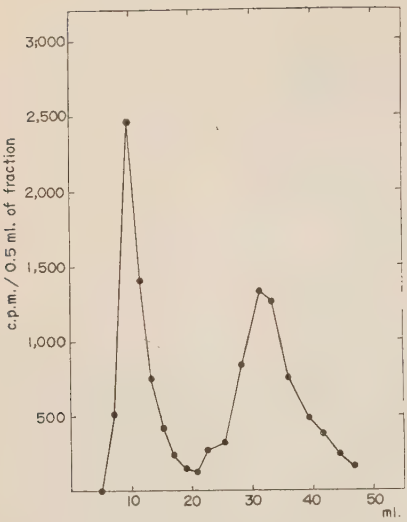

Fig. 3. Partition chromatography of the metabolites of lithocholic acid incubated with guinea pig liver homogenate. Column: 4.5 g. of Hostalene, phase system F (chloroform/heptane=45:5 as stationary phase and methanol/water=165:135 as moving phase)

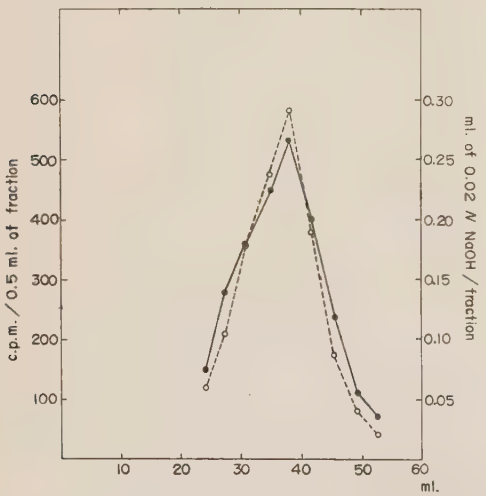

Fig. 4. Rechromatography of the labeled material obtained from the effluents from 26 ml. to 37 ml. shown in Fig. 3 after cocrystallization with unlabeled chenodeoxycholic acid.
Column: 4.5 g. of Hostalene, phase system F (chloroform/heptane=45:5 as stationary phase and methanol/water=165:135 as moving phase)
—— Radio-activity Curve, ... Titration Curve

graphy (Fig. 5).

A typical chromatogram of the metabolites of lithocholic acid incubated with guinea pig liver homogenate is shown in Fig. 3. Apparently it differs from Fig. 1.

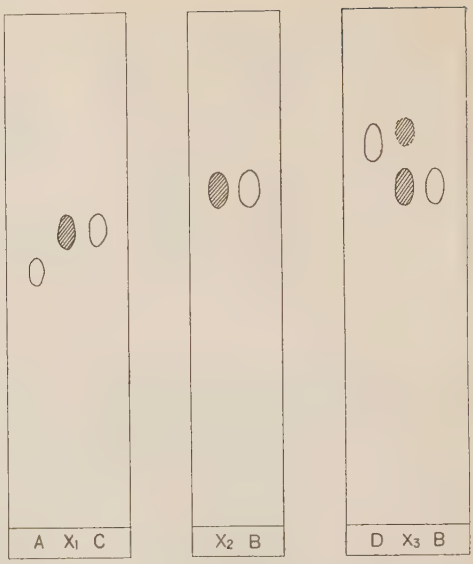

Fig. 5. Paper chromatography and radioautography of the metabolites of lithocholic acid incubated with liver homogenates of rat and guinea pig.
A: hyodeoxycholic acid, B: chenodeoxycholic acid C: 3α,6β-Dihydroxycholanic acid, D: deoxycholic ocid
X_1: The labeled material in the effluents from 17 ml. to 31 ml. (Fig. 1)
X_2: The labeled material in the effluents from 35 ml. to 40 ml. (Fig. 1)
X_3: The labeled material in the effluents from 26 ml. to 37 ml. (Fig. 3)
Shaded area indicates radioactivity.

In this case, the radioactivity curve showed only two peaks and no activity was observed in the region corresponding to 3α,6β-dihydroxycholanic acid, unlike the experiment mentioned above.

It was demonstrated by paper chromatography and subsequent radioautography that the radioactivity in the effluents from 26 ml. to 37 ml. (the peak at 31.3 ml.) of the curve was due mainly to chenodeoxycholic acid and partly to an unidentified dihydroxycholanic acid different from 3α, 6β-dihydroxycholanic acid. Further identification of the former was made by recrystallization of its formyl

ester to a constant specific activity after diluting with unlabeled chenodeoxycholic acid (Table II) and by rechromatography of the free acid obtained through the above formyl ester (Fig. 4).

DISCUSSION

As shown in the present paper, liver homogenates of rat and guinea pig metabolized lithocholic acid differently. This finding seems to give a very important clue to the understanding of the bile acid composition in bile which differs from species to species. Through the extensive investigations carried out by T. Shimizu and his collaborators and others, and also by the present writers, it has been established that individual animal species has its own bile acid composition. Although the present result was obtained with only two animal species, it seems to suggest that such different bile acid compositions as were found in these animal species are due, at least in part, to different activity of the respective liver enzymes.

The stereospecific hydroxylation of bile acid observed in the present work is also important. Lithocholic acid was initially hydroxylated at C-6 or C-7, the resulting hydroxyl group being of axial configuration, and $3\alpha,6\beta$-dihydroxycholanic acid and $3\alpha,7\alpha$-dihydroxycholanic acid were formed by the respective liver enzymes of rat and guinea pig. These results are in accord with the finding that both chenodeoxycholic and deoxycholic acids are hydroxylated at C-6 in bile fistula rat (*11*, *12*). The mechanism of these reactions is under investigation.

SUMMARY

1. Lithocholic acid was incubated respectively with liver homogenates of rat and guinea pig.

2. Lithocholic acid was converted mainly to 3α, 6β-dihydroxycholanic acid and partly to chenodeoxycholic acid by rat liver homogenate.

3. Guinea pig liver homogenate metabolized lithocholic acid mainly to chenodeoxycholic acid and partly to an unidentified dihydroxycholanic acid different from $3\alpha,6\beta$-dihydroxycholanic acid.

The authors wish to thank Mr. T. Hoshita for synthesis of $3\alpha,6\beta$-dihydroxycholanic acid.

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Investigation on Caeruloplasmin

III. Terminal Amino Acids and Sugars of Porcine Caeruloplasmin

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(Received for publication, January 10, 1961)

In previous reports, we described the method of purification and some physicochemical properties of porcine caeruloplasmin, and referred to its sugar components (*1*, *2*). Quite independent of our success in crystallization, Curzon and Vallet (*3*) and Sanders *et al.* (*4*) purified the caeruloplasmin from human serum and Deutsch (*5*) succeeded to crystallize it. However, few work has ever been done on its chemical composition or its primary structure. In this report the experimental results obtained from chemical analysis of crystalline porcine caeruloplasmin dealing with its amino acid composition, terminal amino acids, and sugar components are described.

MATERIALS AND METHODS

Caeruloplasmin—Crystalline caeruloplasmin was obtained from porcine serum by the method reported previously (*2*). The sample was recrystallized twice prior to the analyses. The sample showed a single peak in both ultracentrifugal and paper electrophoretic analysis.

Determination of Sugar Contents—The sugar content was determined colorimetrically by the orcinol method of Fernell *et al.* (*6*) with a Hitachi Spectrophotometer Model EPU-2A.

Acid Hydrolysis of Caeruloplasmin—Approximately 1 mg. of caeruloplasmin was hydrolyzed at 90°C with 1 ml. of 1 N hydrochloric acid for 2 hours. The hydrolyzate was then freed from the acid by evaporation *in vacuo* and dried in a desiccator over sodium hydroxide. The material thus obtained was dissolved in a minimum volume of water and subjected to both paper chromatographic and paper electrophoretic analysis.

Chromatographic Analysis of Acid Hydrolyzate—Paper chromatogrhphy was carried out one-dimensionally or two-dimensionally by ascending method. Aliquots of 0.01 ml. of the concentrated hydrolyzate and/or authentic samples were applied to Toyo No. 50 filter paper, and were developed one-dimensionally with phenol-0.1 per cent ammonia (100 : 23 in weight ratio) or *n*-butanol-acetic acid-water (4 : 1 : 2 in volume ratio). Two-dimensional chromatograms were run with phenol-ammonia as the first solvent and *n*-butanol-acetic acid-water as the second. Aniline-phthalic acid (*7*) or benzidine, and ninhydrin were used to identify the position of the sugars and amino acids, respectively. After the development of the color with aniline-phthalic acid, the chromatograms were examined under the illumination of an ultraviolet lamp to detect fluorescent spots (spots of sugars). Elson-Morgan reaction was used for the detection of amino sugars.

Separation of Sugars by Paper Electrophoresis—Aliquots of acid hydrolyzate of porcine caeruloplasmin or pure authentic samples (glucose, mannose and galactose) were applied to a Toyo No. 50 filter paper, and electrophoresis was carried out with 1 per cent borate buffer.

Enzymatic Hydrolysis with Pronase-P—Approximately 10 mg. of caeruloplasmin in phosphate buffer of pH 7 was hydrolyzed enzymatically with Pronase-P [a protease purified from *Streptomyces griseus* (Kaken Kagaku Co. Ltd.)] for 20 hours at 30°C. The solution after hydrolysis was dialyzed for 20 hours through cellophane membrane against distilled water. The dialysate was then concentrated *in vacuo* and was subjected to chromatographic or electrophoretic analysis.

Enzymatic Hydrolysis with Trypsin—10 mg. of caeruloplasmin in sodium bicarbonate solution of pH 8—9 was digested with crystalline trypsin (Tokyo Tanabe Seiyaku Co. Ltd.) for 12 hours at 30°C. The hydrolyzate was then treated in the same way as described above for the hydrolyzate with Pronase-P.

Separation of Peptide by the Combination Method of Chromatography and Electrophoresis—An aliquot of enzymatic hydrolyzate of caeruloplasmin was applied to a

Tokyo No. 50 filter paper at the middle of a line previously drawn parallel to the bottom of the sheet and was developed chromatographically to the vertical direction with *n*-butanol-acetic acid-water and then electrophoretically to the horizontal direction with pyridine-acetic acid-water (50 : 2 : 948 in volume ratio) of pH 6.5.

Determination of N-Terminal and C-Terminal of Caeruloplasmin—Dinitrophenyl caeruloplasmin (DNP-caeruloplasmin) was prepared according to the method of Levy (*8*). Approximately 20 mg. of DNP-caeruloplasmin was suspended in 1 ml. of 6 *N* hydrochloric acid and kept at 105°C for 12 hours. The solution thus obtained was diluted 6 times with distilled water, and was extracted with ethyl ether. The ether layer was dried *in vacuo* after repeated shaking with 1 *N* hydrochloric acid, and was subjected to two-dimensional chromatographic analysis by themethod of Levy (*8*). The aqueous layers were concentrated *in vacuo* to a small volume, and analyzed by one-dimensional chromatography. *n*-Butanol-acetic acid-water or 1.5 *M* phosphate buffer of pH 6.0 was used as the solvent. C-Terminal of porcine caeruloplasmin was determined by hydrazinolysis reported by Akabori *et al.* (*9*).

Dinitrophenylation of Sugar Containing Peptide—Sugar containing peptide in the hydrolyzate of caeruloplasmim by Pronase-P, which had been separated from other peptides and amino acids by the combination method of chromatography and electrophoresis, was dissolved in 1% trimethylamine solution, and mixed with 0.1 ml. of 5% dinitrofluorobenzene solution in ethanol. The reaction mixture was then left standing for 2 hours in the dark. Excess and non-reacted dinitrofluorobenzene was extracted 3 times from the mixture with ethyl ether. DNP-peptide thus obtained was hydrolyzed by the same method as described above for DNP-caeruloplasmin.

RESULTS AND DISCUSSION

Examination on the paper chromatogram of the acid hydrolyzate revealed the following facts. i) Porcine caeruloplasmin is composed of common amino acids: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, leucines, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine. ii) It contains hexoses and a pentose. The analysis of the chromatogram and electrophoretic pattern of the acid hydrolyzate in comparison with pure authentic samples showed that they are xylose, mannose and glucose, respectively (Figs. 1 and 2). The contents of these sugars in porcine caeruloplasmin determined by the orcinol method were 0.33 per cent of xylose and 1.95 per cent of glucose and mannose. No amino sugar was detected by the Elson-Morgan reaction on the chromatogram.

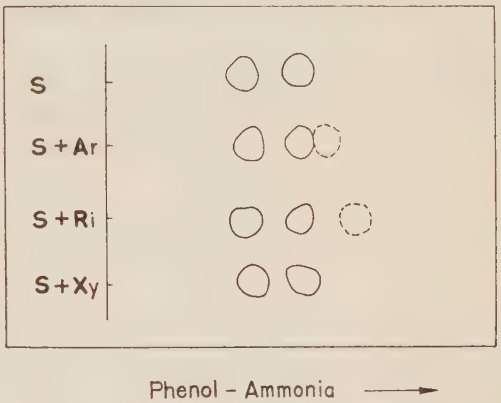

Fig. 1. Separation of sugar components in the acid hydrolyzate of porcine caeruloplasmin by one-dimensional paper chromatography. The abbreviations used are: S, acid hydrolyzate of porcine caeruloplasmin; Ar, arabinose; Ri, ribose; Xy, xylose, respectively.

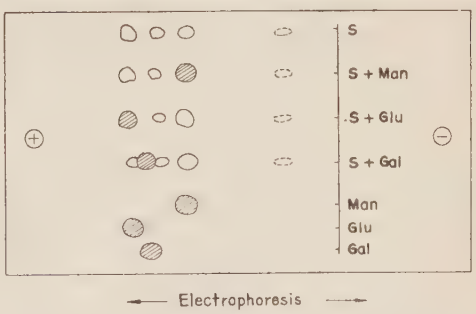

Fig. 2. Separation of sugar components in the acid hydrolyzate of porcine caeruloplasmin by paper electrophoresis. The abbreviations used are: Man, mannose; Glu, glucose; Gal, galactose, respectively.

The analysis by combination method of paper chromatography and paper electrophoresis showed only four kinds of sugar containing oligopeptide or amino acid in the hydrolyzate by Pronase-P which has no specificity in digesting peptide bonds (Fig. 3). On the

other hand, the same analysis of the hydrolyzate by trypsin showed many kinds of sugar containing spots (Fig. 4). These findings suggest strongly that sugar components combine with a few kinds of amino acids in peptide chains. It may be of great interest to determine the locus of sugars more precisely, which may have a close relation with the inhibitory effect of borate ion on the enzymatic activity of porcine caeruloplasmin as will be discussed in the forthcoming paper.

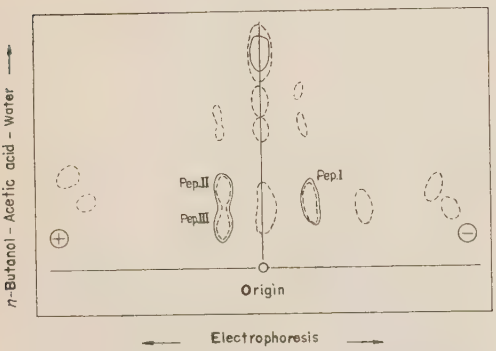

Fig. 3. Separation of peptides and amino acids by combination method of paper chromatography and electrophoresis. Porcine caeruloplasmin was digested with Pronase-P as described in the text. Spots of amino acids and peptides are indicated by dashed circles. Sugar containing spots are circled with solid line.

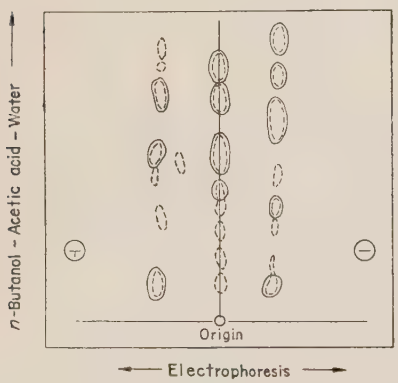

Fig. 4. Separation of peptides and amino acids by combination method of paper chromatography and electrophoresis. Porcine caeruloplasmin was digested with crystalline trypsin as described in tne text.

The chromatogram of the hydrolyzate with Pronase-P shown in Fig. 3 shows three spots of peptide colored with aniline-phthalic acid. These spots were named Pep. I, Pep. II and Pep. III as indicated in the figure. Only Pep. I was separated enough from other peptides and amino acids. It was then eluted from the paper and was analyzed by the following procedures. The sample was hydrolyzed by acid and the hydrolyzate was studied electrophoretically on its amino acid composition. The electrophoretic pattern of the hydrolyzate showed 3 spots of amino acids colored with ninhydrin (Fig. 5).

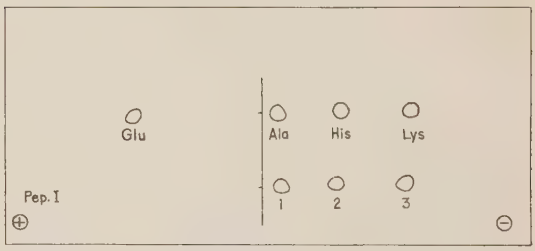

Fig. 5. Separation of amino acids in the acid hydrolyzate of Pep. I by paper electrophoresis. Abbreviations used are: Ala, alanine; His, histidine; Lys, lysine, respectively.

'Spot 1' and 'Spots 2 and 3' are that of neutral and basic amino acids, respectively, as judged from their electrophoretic mobility. 'Spot 2' is positive to Pauly's diazo-reaction indicationg that this is the spot of histidine. 'Spot 3' was identified to be lysine by chromatographic and electrophoretic analyses of the hydrolyzate and authentic samples (lysine and arginine). A part of Pep. I was dinitrophenylated. DNP-Pep. I thus obtained showed a single spot in both chromatographic and electrophoretic analyses. It was then hydrolyzed with acid and the hydrolyzate was separated to ether soluble and water soluble parts. The latter part was analyzed by the combination method of paper chromatography and electrophoresis (Fig. 6). The analysis showed a yellowish colored spot identified as imidazole-DNP-histidine and colorless spots of a free neutral amino acid

and lysine. The free neutral amino acid was found to be alanine. No ε-amino-DNP-lysine was detected in this water soluble part. The ether soluble part of the hydrolyzate of DNP-Pep. I was studied according to the method of Levy. N-Terminal amino acid was found to be threonine. These results elucidated the facts that Pep. I was sugar containing peptide composed of threonine (N-terminal), alanine, lysine and histidine, and that, probably, the sugar was bound to the peptide chain at the ε-amino residue of lysine.

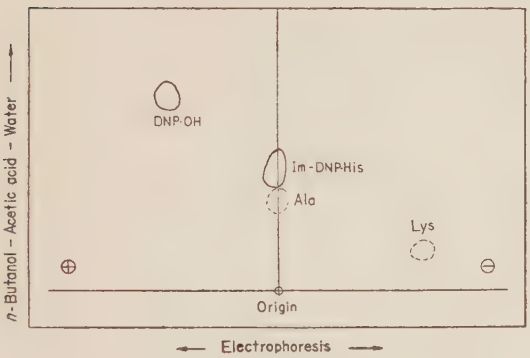

Fig. 6. Separation of hydrolyzed DNP-Pep. I by combination method of chromatography and electrophoresis. The abbreviations used are: DNP·OH, dinitrophenol; Im-DNP-His, imidazole-DNP-histidine, respectively.

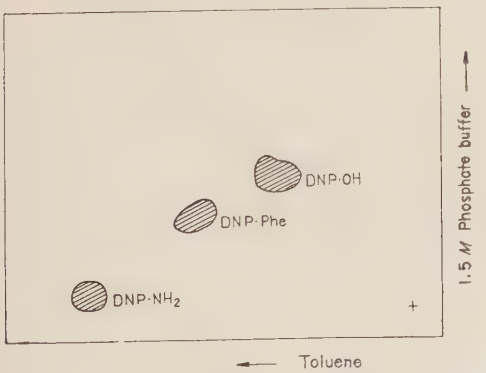

Fig. 7. Separation of DNP-amino acids in the acid hydrolyzate of DNP-caeruloplasmin. The two-dimensional paper chromatography was run with toluene as the first solvent and 1.5 *M* phosphate buffer of pH 6.0 as the second. Abbreviations used are: DNP·NH_2, dinitroaniline; DNP-Phe, dinitrophenylphenylalanine, respectively.

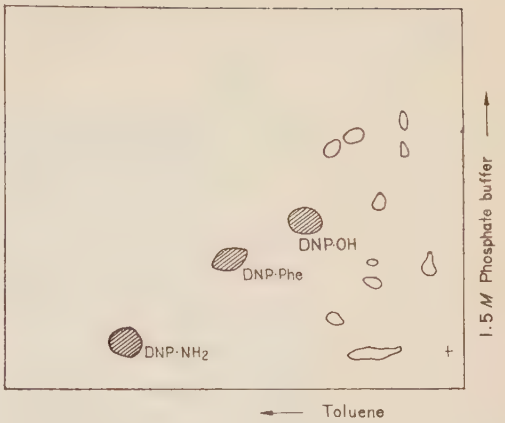

Fig. 8. Separation of carboxy-terminal amino acid.

Both N-terminal and C-terminal amino acids of porcine caeruloplasmin were found to be phenylalanine, which was identified on the chromatogram shown in Figs. 7 and 8.

All these findings which were obtained in this investigation proved that the caeruloplasmin molecule is composed of polypeptide chain(s) which have phenylanine as both amino-terminal and carboxy-terminal amino acids, and that a part of sugar components is probably bound to the ε-amino group of lysine in the peptide chain(s).

SUMMARY

The primary structure of porcine caeruloplasmin was studied by paper chromatography, paper electrophoresis and combination method of paper chromatography and electrophoresis. The molecule is composed of common amino acids as well as three kinds of sugars. Both amino-terminal and carboxy-terminal amino acids were found to be phenylalanine. A sugar containing oligopeptide was isolated after hydrolysis with Pronase-P. The peptide is made up of four amino acids: threonine, alanine, lysine and histidine. It was suggested that the sugar is bound to the ε-amino group of lysine.

The authors wish to express their deep gratitude to Prof. S. Hattori, University of Tokyo and Prof. T. Shibata and Dr. Nagahisa, Tokyo Institute of Technology, for their kind guidance throughout

this work. The authors are also thankful to Mr. Kikuchi for his help in this investigation.

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Investigation on Caeruloplasmin

IV. The Effect of Borate Ion on the Oxidase Activity of Porcine Caeruloplasmin

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(Received for publication, March 9, 1961)

Previously, we succeeded in crystallizing porcine caeruloplasmin, a sugar-containing coppor protein in blood, and described its chemical and physico-chemical properties; terminal amino acids and sugars, $S_{w,20}=7.6S$, $D_{w,20}=4.4\pm0.01\times10^{-7}$ cm^2 sec^{-1}, M.W.$=162{,}000\pm1{,}700$, and ε at 610 mμ $=1.01\times10^4$ (*1–3*). Borate has an inhibitory effect on the activity of various enzymes such as glucosulfatase (*4*), alkaline phosphatase, xanthine oxidase *etc.* (*5*). As is well known, borate has a strong affinity with carbohydrates, so that some of these inhibitory actions may be accounted for by combination between borate and two hydroxyl group of glycol type in the enzyme molecules. In 1951, Holmberg and Laurell (*6*) and, more recently, Curzon (*7*) studied the inhibition of its oxidase activity by various monovalent anions. However, the effects of borate on the activity have not ever been reported. Our previous finding that porcine caeruloplasmin contains approximately 2% sugars, glucose, mannose and xylose, let the author to the present study on the effect of borate upon the oxidase activity of porcine caeruloplasmin. Herein are reported the results obtained in the experiments together with some deduction drawn from them as to the amino acid residues chelating the copper atom in the caeruloplasmin molecule.

MATERIALS AND METHODS

Enzyme—Crystalline porcine caeruloplasmin was prepared according to the procedure described in the previous paper (*2*). The concentration of the enzyme was determined spectroscopically with aid of the ε value of 1.01×10^{-4} at 610 mμ.

Assay—One ml. of a caeruloplasmin solution was mixed with 4 ml. of 0.1 *M* acetate buffer of pH 6.0 and 4 ml. of an inhibitor solution of the same pH*, and kept at 30°C for 5 minutes before the activity measurement. To the sample solution thus obtained, was added 1 ml. of 0.1 *M* *p*-phenylenediamine solution of pH 6.0, and then aliquots of 2 ml. of the reaction mixture was acidified every one minute with 2 ml. of 10% sulfuric acid to stop the enzymic reaction. The rate of the formation of a colored oxidized product in the aliquots of the sample was determined spectroscopically by measuring the absorbance change at 405 mμ with a Hitachi spectrophotometer, model EPU-2A. The pH of the *p*-phenylenediamine dichloride solution was adjusted to a desired value with sodium hydroxide before adding it to a sample solution.

RESULTS

A preliminary experiment was carried out to observe the pH-dependency of the oxidase activity of caeruloplasmin, and the result is illustrated in Fig. 1. The substrate concentration 10^{-2} *M* adopted in the measurement was large enough to saturate the rate of oxidation and to make the rate proportional to enzyme concentration. Although the buffering capacity of 0.04 *M* acetate in the reaction mixture was not sufficient in the range of pH 6.2–6.9, no change of pH was detectable during the measurement of activity. From the curve in Fig. 1, the optimum pH value for the activity was found to be 6.0. This pH value is slightly higher than the value, 5.8, which was obtained for human caeruloplasmin by Curzon (*7*). All the experiments described below were conducted at this optimum pH of 6.0.

The pK value estimated from the left-hand side of the pH-dependency curve in Fig. 1 was 5.8 which is in approximate agree-

* pH of the solution was adjusted by HCl.

ment with the pK value of imidazole residue in the protein. One may, therefore, infer that the dissociation of proton from the positively charged nitrogen in the imidazole residue caused the activation as oxidase between pH 4.0 and 6.0 shown in the figure.

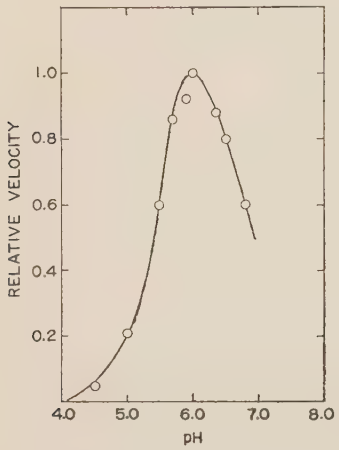

FIG. 1. Oxidase activity of porcine caeruloplasmin as a function of pH. The reaction mixture contains 0.01 M p-phenylenediamine and $8\times10^{-7}M$ caeruloplasmin in 0.04 M acetate buffer.

The time course of the absorbance change in the reactions with $(1—50)\times10^{-3}M$ borate as the inhibitor is shown in Fig. 2, from

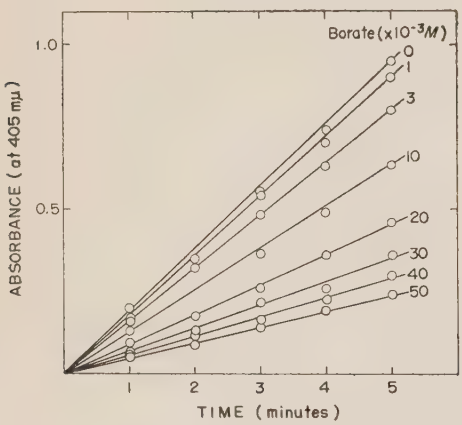

FIG. 2. Effect of borate on the rate of oxidation by $2.5\times10^{-7}M$ caeruloplasmin at pH 6.0.

which the degree of inhibition, H, defined by equation (1), was estimated.

$$H=(v_0-v)/v_0 \qquad (1)$$

In the above equation, v and v_0 are the rate of reaction with and without borate. The value of H is plotted against the logarithm of borate concentration in Fig. 3,

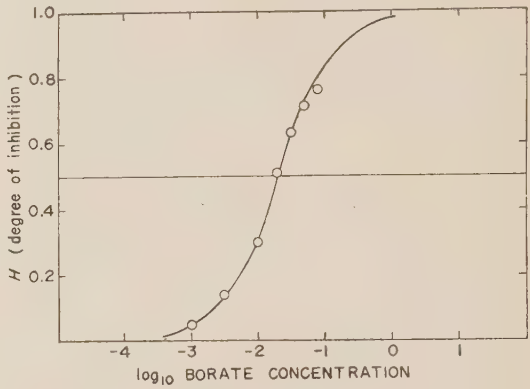

FIG. 3. The relationship between borate concentration and the degree of inhibition, H.

which shows a sigmoid curve of the first order. The order of the curve indicates that the combination of one borate ion with the active center or the neighboring residues in the caeruloplasmin molecule results in complete inhibition of the activity. Thus, the borate concentration for 50% inhibition is equal to the equilibrium constant, K shown below:

$$K=\frac{[E]\,[G]}{[EG]} \qquad (2)$$

where [E] and [G] stand for molar concentrations of enzyme and its inhibitor, borate ion, and [EG] is the concentration of the complex between them having no activity. The value of K estimated from the curve in Fig. 3 was $1.92\times10^{-2}M$. Böeseken (*8*) determined the equilibrium constants for the reaction between borate and various sugars by observing the changes of electric conductivity and optical rotatory power of their mixtures, and obtained their K values ranging between 0.8×10^{-2} and $3.1\times10^{-2}M$. The value of K observed for caeruloplasmin lies in the range of K obtained by Böeseken, suggesting that the reaction responsible for the inhibition is the combination of borate with a sugar or sugars in the caeruloplasmin molecule.

The further analysis was made as to the

mode of inhibition by borate. The linear relationship obtained by Lineweaver-Burk's way of plotting, is shown in Fig. 4,

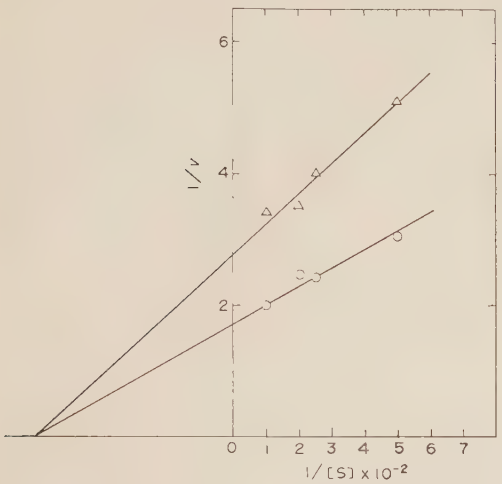

Fig. 4. The relation between reciprocal velocity, 1/v, and reciprocal *p*-phenylenediamine concentration, 1/[S] with and without borate; —○—; no inhibitor, —△—; $1.0\times10^{-2}M$ borate.

in which [S] is the concentration of substrate, *p*-phenylenediamine. Two straight lines showing the relation with and without borate in the figure intersect with each other at a point on the abscissa. Therefore, the inhibition by borate is non-competitive type, being different from the inhibition of caeruloplasmin by monovalent anions such as acetate or chloride which was confirmed to be anti-competitive type by Curzon (*7*). This fact suggests that the inhibition by borate is not the direct combination of borate with the active center or the copper atom in the caeruloplasmin molecule but is the combination with any other residue being located near the active center.

The inhibitory effect of borate on the oxidase activity was found to be of reversible nature. The results in the reversibility test are shown in Fig. 5, which indicates the time courses of absorbance increase for three different samples. Solid and open circles are the absorbance readings for the sample containing $1.4\times10^{-7}M$ caeruloplasmin mixed with 2×10^{-2} and $5\times10^{-2}M$ borate, respectively. A more concentrated caeruloplasmin solution was pretreated with $5\times10^{-2}M$ borate, and was then diluted with water so as to obtain

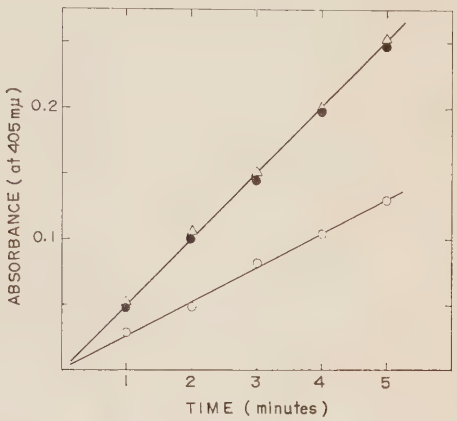

Fig. 5. Reversibility of the combination of borate with $1.4\times10^{-7}M$ caeruloplasmine. —●—; absorbance change observed with $2\times10^{-2}M$ borate, —○—; absorbance change observed with $5\times10^{-2}M$ borate, —△—; absorbance change of the sample pretreated with $5\times10^{-2}M$ borate but observed with $2\times10^{-2}M$ borate.

the same concentration of caeruloplasmin and $2\times10^{-2}M$ borate in the measurement of its activity. The absorbance readings for this sample are shown by triangles in the figure, which completely agree with the data shown by solid circles. The reversibility thus elucidated of the combination of borate becomes the experimental basis for equation (2) assumed for the analysis of the inhibitory action.

DISCUSSION

In discussing the oxidase activity of caeruloplasmin, of great importance is the copper-linked amino acid residue of the molecule. It was clarified in the previous study (*1*) that the blue color of caeruloplasmin reappeared when colorless and copper-free caeruloplasmin obtained by treatment with cyanide was dialyzed against 'cuprous' chloride solution. The same experiment by dialysis with 'cupric' ion showed no reappearance of the blue color. These results obtained with cuprous and cupric ion sug-

gests that the copper atom in the caeruloplasmin is in cuprous state, although the electronic state of the cuprous atom in the molecule may be more or less perturbed by the influence of the copper-linked residue or residues. Recently, Orgel (*9*) scrutinized and compared the affinities of cuprous and cupric ions with various amino acid residues of protein and concluded that cuprous ion has a strong affinity with imidazole or sulfhydryl group. The possibility of its combination with sulfhydryl group in the caeruloplasmin molecule can be eliminated, considering the following three facts obtained in the present and previous studies. i) The pH-activity curve in Fig. 1 between pH 4.0 and 6.0 can be well accounted for by the dissociation of proton from the imidazole residue of the molecule. ii) The chemical analysis of caeruloplasmin showed the absence of sulfhydryl group, whereas the oligopeptide obtained by enzymic digestion of caeruloplasmin was found to be made up of a sugar or sugars and 4 amino acids including 'histidine'. iii) Copper atom in the caeruloplasmin molecule is exchangeable with Cu^{64} in the presence of the substrate for its oxidase activity (*10*). However, no significant effect of -SH inhibitor on the oxidase activity of caeruloplasmin was observed (*11*). These experimental facts bear evidence that the copper-linked residue is the imidazole residue of the caeruloplasmin molecule.

Based upon the inference described above, the inhibition by borate on the oxidase activity of caeruloplasmin may be explained in the following manner. As was cited above, borate ion is known to have a strong affinity with sugars, and its combination occurs at two hydroxyl groups of glycol type of sugar molecules. For the following reasons, the inhibition by borate observed in this study seems to be due to its combination with sugars contained in the caeruloplasmin molecule. i) The observed values of the equilibrium constant K agree fairly well with the values obtained previously for the reactions between borate and various sugars. ii) The caeruloplasmin molecule contains 2% sugars; glucose, mannose, and xylose, and oligopeptide obtained from caeruloplasmin also contains a sugar or sugars. The mode of inhibition by borate was proved to be non-competitive type. This excludes the possibility of direct combination of borate with the copper atom of the molecule, and supports an indirect effect of borate on the oxidase activity, possibly through a sugar molecule. Thus, a model, which may be postulate for the active center of oxidase activity, would be a cuprous atom chelated with an imidazole residue or residues and a sugar located in its neighborhood.

The fact that the oligopeptide described above contains both a sugar and imidazole residue in the small molecule suggests the possibility of interaction between the sugar and imidazole residue, so that the combination of borate with the sugar may result in the inhibition of the activity. The further experiments are contemplated to elucidate the more detailed structure of the active center.

SUMMARY

The oxidase activity of crystalline porcine caeruloplasmin was studied spectroscopically with *p*-phenylenediamine as the substrate. Borate ion inhibited the oxidase activity and the inhibition was found to be of reversible nature. The functional relationship obtained between borate concentration and the degree of inhibition indicated that the inhibition is the non-competitive type and occurs by combination of a borate ion with a sugar contained in the caeruloplasmin molecule. The structure of the active center near the copper atom of the molecule is discussed, refering the previous results described in this series of reports.

The author wishes to express his deep gratitude to Prof. S. Hattori, University of Tokyo, and Prof. T. Sato, Prof. K. Shibata and Dr. Nagahisa, Tokyo Institute of Technology, for their valuable advice and interest throughout this investigation. He is also thankful to Dr. Kanazawa, Mr. Kaya and Mr. Kikuchi for their help in carrying out this study.

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Glutamic Acid Formation from Glucose by Bacteria

VI. Metabolism of the Intermediates of the TCA Cycle and of the Glyoxylate Bypass in *Brevibacterium flavum* No. 2247*

By Isamu Shiio, Shin-ichiro Ôtsuka and Masahiro Takahashi

(*From the Central Research Laboratory of Ajinomoto Co., Inc., Kawasaki*)

(Received for publication, January 31, 1961)

From the results shown in the previous papers, there seems little doubt that terminal respiration in *Brevibacterium flavum* No. 2247 occurs *via* the modified TCA cycle with an additional glyoxylate bypass (*1*, *2*). Thus, all the enzymes of the TCA cycle and of the glyoxylate bypass, except for α-ketoglutaric dehydrogenase, have been found to be present in the cell-free extracts (*1*, *3*). Moreover, in accordance with the operation *in vivo* of condensing enzyme, aconitase, and isocitric dehydrogenase, inhibition experiments (*4*) have shown that glucose is metabolized aerobically by the resting cells to form αKG *via* pyruvate and citrate successively. Pyruvic oxidase, one of the key enzymes for pyruvate to enter the TCA cycle, has also been demonstrated in the cell extracts. From the isotopic tracer experiments, it has been shown that the incorporation of C^{14} of $C^{14}O_2$ into α-carboxyl carbon of the glutamate formed from glucose as well as the distribution of C^{14} in the glutamate formed from acetate-1- and -2-C^{14} can be well explained by the cycle as described above (*2*, *5*). Furthermore, kinetic analysis of the incorporation of C^{14}-acetate into the intracellular organic acids of the cycle gave the results indicating the operation of the cycle in the intact cells (*6*).

The present paper deals with the metabolism of the intermediates of the TCA cycle and of the glyoxylate bypass in the resting cells.

METHODS

Chemicals—Glyoxylic acid, oxaloacetic acid, L-malic acid, pyruvic acid and αKG were purchased from the California Corporation for Biochemical Research.

Chemical Determination—Pyruvate and αKG in their mixture were determined as their DiNPH by the slightly modified method of Friedemann and Haugen (*7*). Oxaloacetate, pyruvate and αKG in their mixture were measured colorimetrically after the chromatographic separation of their DiNPH (*8*, *9*) on a filter paper which were developed with *n*-butanol-ethanol- 0.5 *N* NH_4OH (7 : 1 : 2) by the descending technique untill they were completely separated from each other. Methods for the determination of citrate and of malate were essentially the same as those of Natelson *et al.* (*10*, *11*) and of Goodban and Stark (*12*). Column chromatographic separation of non-volatile acids was performed according to the method of Busch *et al.*, in which non-volatile carboxylic acids were separately eluted from anion exchange resins with increasing concentrations of volatile acids (*13*). Paperchromatographic solvents used were *n*-butanol-90% formic acid -water (4 : 1.5 : 1) and phenol-90% formic acid -water (500 g. : 13 ml. : 167 ml.) for non-volatile acids. The method for the preparation of washed cell suspension as well as methods for the determination of the nitrogen content of cells, of glyoxylate in the presence of αKG, and of glutamic acid was described previously (*3*, *14*).

RESULTS

Interconversion between Fumarate and Malate—When sodium fumarate was incubated with the resting cells, it was converted to malate which was isolated from the reaction mixture.

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* The study was partially reported at 33rd General Meeting of the Japanese Biochemical Socity (1960).

The abbreviations used through this paper include αKG, α-ketoglutaric acid; DiNPH, 2, 4-dinitrophenylhydrazone; FAc, monofluoroacetate.

Fig. 1 shows the chromatographic elution pattern, where the abscissa scale refers to the tube number after the appearence in the eluate of glutamate added as an indicator for the beginning of the elution of non-volatile acids. Two peaks (I, II in Fig. 1) were obtained; the first was malate, and the second fumarate. Malate was indentified by its paperchromatographic behaviour with the solvent systems as described in "methods", and by the absorption spectrum of the colored reaction product with 2, 7-dihydroxynaphthalene (*12*) (Fig. 2). Fumarate was also identified paperchromatographically.

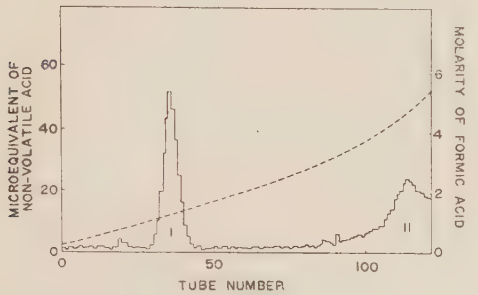

Fig. 1. Chromatographic elution pattern of the organic acids formed from fumarate by the resting cells.

Reaction mixture contained 40 μmoles of sodium fumarate and 0.65 mg. N of resting cells in a final volume of 1 ml. of 0.1 *M* phosphate buffer, pH 7.0. Incubated aerobically at 37°C for 5 hours. 15 ml. of the mixture was concentrated to 4 ml., and placed on a Dowex 1X8 (formate form) column, 27×0.8 cm. (*13*). —, microequivalent of non-volatile acid in the eluate; – – –, molarity of formic acid used for elution.

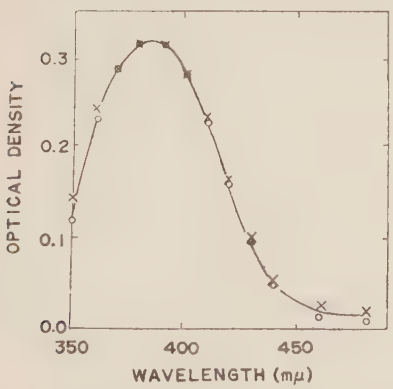

Fig. 2. Absorption spectra of the reaction products of 2, 7-dihydroxynaphthalene with malic acid (—○—) and with acid in peak I of Fig. 1 (—×—) (*12*).

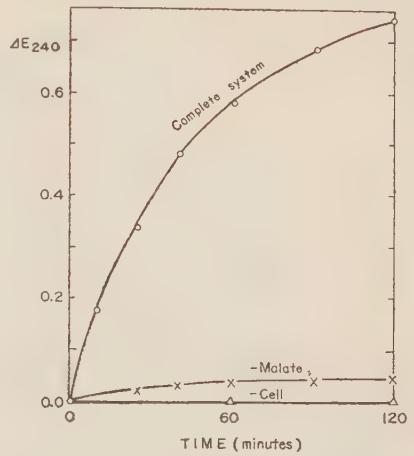

Fig. 3. Formation of fumarate from L-marate by resting cell.

Complete system contained 40 μmoles of sodium L-malate and 0.074 mg. N of resting cells in a final volume of 1 ml. of 0.1 *M* phosphate buffer, pH 7.0. Incubated at 37°C. Then, the reaction mixture was centrifuged and the supernatant solution was analyzed after tenfold dilution.

Formation of fumarate from L-malate by the resting cells was demonstrated by the spectrophotometric measurement of an increase of absorption at 240 mμ due to the double bond of the fumarate formed (Fig. 3). Paperchromatographic examination also indicated the formation of fumarate.

Formation of Oxaloacetate from L-Malate and from Succinate—When L-malate or succinate was incubated aerobically with the resting cells in the presence of semicarbazide, a considerable amount of oxaloacetate as well as a small amount of αKG and of pyruvate was found in the reaction mixture. Oxaloacetate was identified by the paperchromatographic behaviour and characteristic absorption spectrum (Fig. 4, 5) of its DiNPH.

Effect of Inhibitors on the Oxidation of Succinate—As shown in Table I (a), L-glutamate or αKG was accumulated in the oxidation

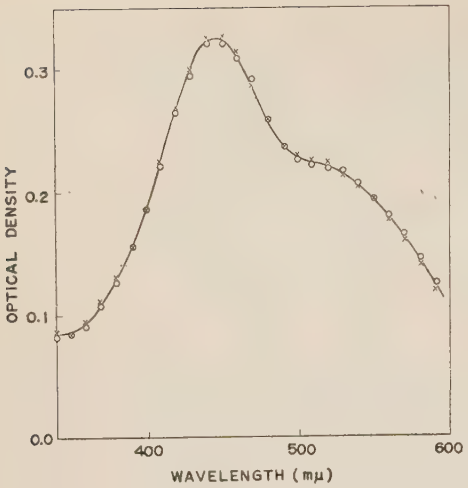

Fig. 4. Absorption spectra of 2,4-dinitrophenylhydrazones of oxaloacetate (—○—) and of product (—×—) accumulated when succinate was oxidized by resting cells in the presence of semicarbazide.
Solvent: 1 *N* NaOH

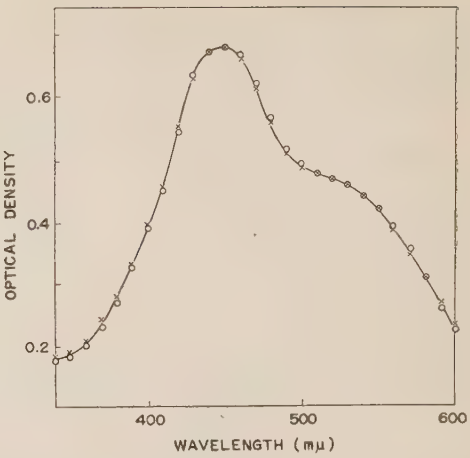

Fig. 5. Absorption spectra of 2,4-dinitrophenylhydrazones of oxaloacetate (—○—) and of product (—×—) accumulated when L-malate was oxidized by resting cells in the presence of semicarbazide.
Solvent: 1 *N* NaOH

Table I

Inhibitors and Products in the Oxidation of Succinate (a)

NH_4-salt	Inhibitor		Concentration of product after 5 hrs. (μmole/ml.)			
	$NaAsO_2$	$CH_2FCOONa$	Pyruvate	Citrate	αKG	L-Glutamate
+	−	−	0.7	0.0	0.7	7.8
+	−	+	0.2	1.4	0.3	0.5
+	+	−	5.1	0.0	0.1	−0.1
+	+	+	5.0	0.0	0.0	0.0
−	−	−	0.0	0.0	6.5	0.5
−	−	+	0.3	1.4	0.4	−0.1
−	+	−	4.5	0.0	1.1	0.1
−	+	+	3.9	0.0	0.7	0.1

Inhibitors and Products in the Oxidation of Succinate (b)

Inhibitor		Concentration of product after 5 hrs. ($\times 10^{-1}$ μmole/ml.)	
$NH_2NHCONH_2$	$CH_2FCOONa$	Oxaloacetate	Citrate
+	+	6.8	2.2
+	−	5.7	0.0
−	+	−0.2	4.9
−	−	−0.5	0.3

Reaction mixtures contained 40 μmoles of sodium succinate, 50 μmoles of inhibitor, 30 μmoles of NH_4Cl (the cases indecated by + in (a)), and 0.37 mg. N (a) or 0.50 mg. N (b) of resting cells in a final volume of 1 ml. of 0.1 *M* phosphate buffer, pH 7.0. Incubated aerobically at 37°C for 5 hours.

of succinate by the resting cells. Under the same conditions, the addition of arsenite and of FAc, however, yielded pyruvate and citrate, respectively, and prevented the formation of L-glutamate or of αKG. The presence of both inhibitors caused also the accumulation of pyruvate. Under the conditions, where citrate formed aerobically from succinate in the presence of FAc by the resting cells, the addition of semicarbazide caused the decrease of the citrate as well as the oxaloacetate accumulation, the level of which was higher than that observed in the presence of semicarbazide alone as inhibitor (Table I (b)).

Effect of Inhibitors on the Oxidation of Malate—Inhibition experiments using L-malate as a substrate were carried out in the same manner as described above (Table II). Although a small amount of αKG was found in the absence of the inhibitors, the presence of arsenite and of FAc resulted in the accumulation of pyruvate and of citrate, respectively. When both of them were present in the reaction mixture, the results were similar to that observed in the presence of arsenite alone. The presence of semicarbazide, regardless of the presence of other inhibitors, caused the accumulation of oxaloacetate. On the other hand, accumulation of citrate in the presence of FAc was decreased by the addition of semicarbazide to about one fourth. In the presence of semicarbazide, further addition of arsenite and or of FAc caused the accumulation of pyruvate.

Metabolism of Glyoxylate—The resting cells catalysed the oxidation of glyoxylate and the amount of CO_2 evolved reached to a level expected from the complete oxidation of the substrate, 2 moles per mole of glyoxylate.

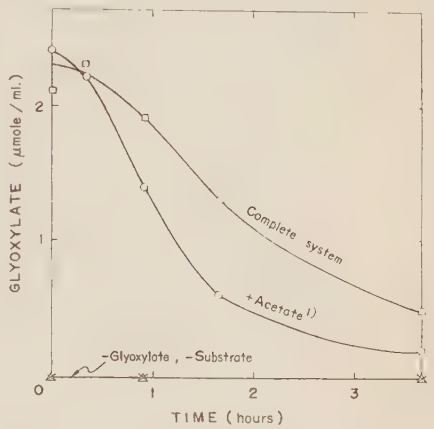

Fig. 6. Oxidative metabolism of glyoxylate by resting cells and effect of acetate thereon.

Complete system contained 2 μmoles of sodium glyoxylate and 0.37 mg. N of resting cells in a final volume of 1 ml. of 0.1 *M* phosphate buffer, pH 7.0. Incubated aerobically at 37°C.

1) 8 μmoles of sodium acetate was added to 1 ml. of the reaction mixture.

Table II

Inhibitors and Products in the Oxidation of L-Malate

Inhibitor			Concentration of product after 5 hrs. (μmole/ml.)		
$NH_2NHCONH_2$	$NaAsO_2$	CH_2 FCOONa	Oxaloacetate	Pyruvate	Citrate
−	−	−	0.2	−0.3	0.06
−	−	+	0.2	2.3	0.78
−	+	−	0.8	1.2	0.00
−	+	+	0.2	1.3	0.02
+	−	−	3.4	0.4	0.07
+	−	+	3.5	1.8	0.21
+	+	−	2.8	2.2	0.01
+	+	+	3.7	1.8	0.02

Reaction mixtures contained 40 μmoles of sodium L-malate, 50 μmoles of inhibitor, and 0.50 mg. N of resting cells in a final volume of 1 ml. of 0.1 *M* phosphate buffer, pH 7.0. Incubated aerobically at 37°C for 5 hours.

Fig. 6 demonstrates the aerobic consumption of glyoxylate by the resting cells, which seemed to be accelerated by the addition of acetate. Higher concentration of the substrate inhibited strongly the oxidation by the resting cells, for example, Qo_2 at 20 μmoles glyoxylate per ml. was about one tenth of that at 2 μmoles per ml.

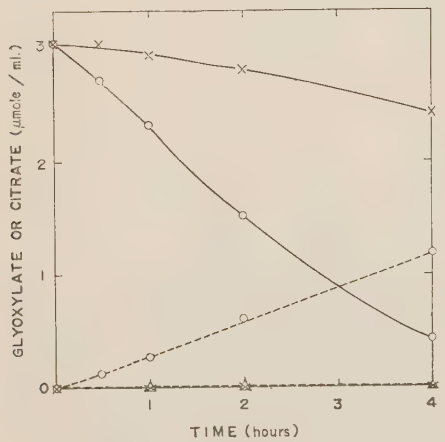

Fig. 7. Anaerobic metabolism of glyoxylate by resting cells in the presence of succinate and formation of citrate.

Complete system of reaction contained 3 μmoles of sodium glyoxlate, 8 μmoles of sodium succinate, and 0.24 mg. N of resting cells in a final volume of 1 ml. of 0.1 *M* phosphate buffer, pH 7.0. Incubated anaerobically in a Thumberg tube at 37°C. ○, complete system; ×, —succinate; △, —glyoxylate; —, glyoxylate; ---, citrate.

Under the anaerobic dondition, the rate of glyoxylate consumption was very low, which was raised by the addition of succinate. Only in the latter case, the formation of citrate was observed (Fig. 7).

DISCUSSION

Succinate was metabolized aerobically by the resting cells to form αKG or glutamate. The inhibition experiments suggest that oxaloacetate, as well as pyruvate and citrate, is an intermediate in the oxidation of succinate, and the probable metabolic sequence of succinate is as follows:

Succinate→pyruvate, oxaloacetate→
citrate→αKG→glutamate.

The results are quite similar to those obtained in the oxidation of glucose (*4*). Another inhibition experiments with L-malate suggest that the three compounds mentioned above are intermediates also in the oxidation of malate. Since succinic dehydrogenase had beed demonstrated in the cell extract (*3*) and malate was found to be formed from fumarate by the resting cells, following metabolic sequence of succinate can be constructed from these results:

Succinate→fumarate→malate→oxaloacetate,
pyruvate→citrate→αKG→glutamate.

All of the enzymes involved in the above metabolic pathway have been actually demonstrated in the cell-free extracts (*1*, *3*). In this organism two possible reactions concerning pyruvate formation have been found, that is, the oxidative decarboxylation of malate catalysed by malic enzyme and the decarboxylation of oxaloacetate catalysed by oxaloacetic decarboxylase. The results obtained from the inhibition experiments cannot tell reasonably which of these alternatives is the main reaction of pyruvate formation.

Glyoxylate was oxidized completely with formations of 2 moles of CO_2 per mole of the substrate. Effect of acetate on the glyoxylate consumption suggests the operation of malate synthetase (*1*, *15*) *in vivo* and consequently the following cyclic metabolic pathway for the complete oxidation of glyoxylate:

Glyoxylate→malate→oxaloacetate→pyruvate→acetylCoA—
↑ +CO_2 +CO_2

The same pathway as described above has been postulated first by Kornberg and Sadler (*16*) for the oxidation of glyoxylate in *E. coli*. Moreover, the evidence for the presence of all the enzymes related to the above cyclic pathway has been reported previously (*1*, *3*, *4*). It should be noted that

the C^{14} distribution in the glutamate formed from C^{14}-acetate as well as the formation of a considerable amount of CO_2 in the oxidation of acetate has been well explained by postulating the above described pathway for the degradation of malate or oxaloacetate (*5*).

Under the anaerobic condition, glyoxylate was metabolized by the resting cells, only when succinate was added. In the latter case, the formation of citrate occurred indicating the operation of isocitratase (*17*, *18*) *in vivo*. Therefore, following pathway would be suggested for the anaerobic formation of citrate from glyoxylate and succinate:

Glyoxylate + succinate → isocitrate → *cis*-aconitate → citrate.

Evidence has been obtained previovsly for the presence of isocitratase and of aconitase in the cell extracts (*3*).

When three pathways described above for the metabolism of the intermediates of the TCA cycle and of the glyoxylate bypass were conbined with each other, a cyclic pathway shown in Fig. 8 can be constructed, which is the same as that postulated previously for the glutamate formation from glucose as well as from acetate in this organism (*1*, *2*).

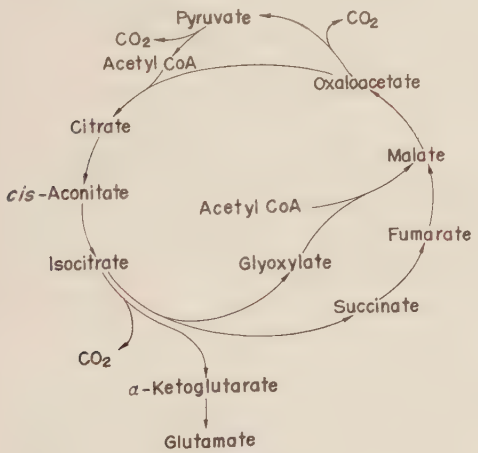

Fig. 8. Probable pathway for the terminal respiration in *Brevibacterium flavum* No. 2247.

In other words, the results obtained with the intact cells support strongly the pathway postulated previously for the glutamate formation.

SUMMARY

1. When succinate was incubated aerobically with the resting cells of *Brevibacterium flavum* No. 2247, α-ketoglutarate, or glutamate in the presence of ammonium salts, was accumulated. The addition of semicarbazide, of arsenite, and of monofluoroacetate caused the accumulation of oxaloacetate, of pyruvate, and of citrate, respectively, instead of α-ketoglutarate. Similar results were obtained with the oxidative metabolism of L-malate, which was found to be formed from fumarate. From the inhibition experiments, following metabolic sequence of succinate was suggested: succinate/fumarate/malate/oxaloacetate, pyruvate/citrate/α-ketoglutarate/glutamate.

2. When glyoxylate was incubated aerobically with the resting cells, 2 moles of CO_2 per mole of glyoxylate was obtained, indicating the complex oxidation. The reaction was accelerated by the addition of acetate. The anaerobic metabolism of glyoxylate was observed only in the presence of succinate, with the formation of citrate.

The authors are indebted to Dr. H. Oeda, Mr. S. Motozaki, and Mr. T. Tsunoda of our laboratory for the helpful advices and encouragement.

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Studies on an *in Vitro* Catalase Inhibitor from Silkworm Blood

II. Fractionation of the Inhibitor

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In a previous paper (*1*), report has been made on the *in vitro* action of a catalase inhibitor found in silkworm blood plasma. The present paper deals with the procedures devised for the purification of the inhibitor. The characteristic properties of the purified catalase inhibitor are also described.

EXPERIMENTALS

The material and the experimental methods are essentially the same as those described in the previous paper (*1*). Boiled extract of blood plasma of silkworm larvae (BE) was used as a source of the catalase inhibitor.

The method for assaying the inhibiting activity was as follows: a mixture, consisting of 0.5 ml. 20 μg./ml. crystalline cow liver catalase; 1 ml. 0.067 *M* phosphate, pH 6.8; 0.5 ml. inhibitor solution; 0.2 ml. 6.25×10^{-3} *M* KNO_3 and 0.3 ml. distilled water (total 2.5 ml.), was preincubated at 0°C for 1 hour. A 0.5 ml. aliquot taken from this mixture was used for the assay of catalatic activity. Potassium nitrate was omitted when non-purified BE was used as inhibitor.

The inhibitor unit was defined as the quantity of inhibitor required to suppress the catalatic reaction by 50 per cent under the experimental conditions described above.

RESULTS

Paper Chromatography of Boiled Extract of Blood Plasma—Attempts were made to isolate the inhibitor by paper chromatography. The boiled extract (BE) was concentrated to 5-fold and ascending chromatography was carried out using various solvent mixtures (see Table I). After removing the solvent, a solution of crystalline cow liver catalase (40 μg. per ml. 0.03 *M* phosphate, pH 6.8) was sprayed onto the paper, and allowed to stand for about 10 minutes. The paper was then sprayed with 1 *M* hydrogen peroxide and finally immersed in 2 *N* sulfuric acid containing titanium sulfate. The inhibitor appeared as a yellow spot, indicating a higher concentration of peroxide remaining as a result of suppressed reaction at the spot* (Table I). It was found that the point on chromatogram corresponding to the inhibitor spot gave always a strong positive reaction (blue) with diphenylamine sulfuric acid (*2*). The R_f values obtained with various solvent mixtures coincided with the corresponding values for nitrate.

Table I

Rf Values for Inhibitor and Nitrate in Boiled Extract of Silkworm Blood Plasma (BE)[1]

Solvent	R_f	
	BE	Nitrate
Phenol, saturated with water	0.22	—
Butanol-acetic acid-water (4 : 1 : 1)	0.27	0.27
Butanol-ethanol-water (2 : 1 : 1)	0.36	0.36
Butanol-ethanol-1% NH_3 (2 : 1 : 1)	0.48	0.49
Propanol-water (2 : 1)	0.56	—
Propanol-1% NH_3 (2 : 1)	0.62	0.63

1) Toyo No. 50 filter paper, by ascending method. Detection of spots by enzymic method (see text) and by diphenylamine-H_2SO_4 (*2*).

A quantitative analysis of boiled extract with respect to nitrate and nitrite (by the method of Nicholas and Nason (*3*)) was

* Several weak yellow spots also tended to appear on the chromatogram on treatment with titanium sulfate. These points are inferred to be due to the presence of various amino acids in the test material, since they coincided with positive spots which developed on application of ninhydrin.

then carried out to discover contents of $2.0\sim2.5\times10^{-3}\,M$ nitrate and $5\sim8\times10^{-5}\,M$ nitrite per liter. When the indicated concentrations of these ions were added to the solution of catalase, there was only a slight inhibition; 13 per cent with nitrate and 5 per cent with nitrite (*cf.* Stern (*4*), Agner and Theorell (*5*)). The major part, at least, of the inhibiting action of BE can not, therefore, be accounted for by the action of these inorganic ions. A search for the genuine inhibitor in silkworm blood plasma was then made with the aim of obtaining the substance in a purified state.

Isolation of the Inhibitor from Silkworm Blood Plasma—The catalase inhibitor of silkworm blood was effectively purified by the following procedures: Blood plasma was collected from about 6,000 silkworm larvae (5 th instar). The blood plasma collected from 500~1,000 larvae was heated in a boiling water bath for 20~30 minutes, and stored at 0°C. The heat-treated plasma (total 1,600 ml.) was filtered through a Büchner funnel with suction, the clotted mass on the funnel being extracted several times with each 500 ml. of boiling water.

The catalase inhibitor was precipitated from the combined extract (4,000 ml.) by adding 200 ml. of 50 per cent (w/v) silver nitrate. (The inhibitor was precipitated also by lead subacetate, but not by mercuric or barium acetate.)

The precipitate was centrifuged off, washed with a small amount of cold water, and dessiccated *in vacuo* (dry weight 34.4 grams).

Five gram portions of the silver salt powder were used for further purification. The finely ground dry powder was washed twice with each 30 ml. of cold water, and suspended in 50 ml. of 0.5 *N* HCl. Hydrogen sulfide was passed through the suspension for 30 minutes with addition of a drop of octyl alcohol to prevent foaming. The silver sulfide formed was centrifuged off and washed twice with each 10 ml. of 0.5 *N* HCl. Sulfur dioxide gas was bubbled through the combined solution, and 1 g. of Norit was added to adsorb inactive colored material. The Norit was removed by filtration and washed with a small amount of water. The filtrate was added to the main filtrate, and the solution concentrated under reduced pressure at 45°C, in a stream of hydrogen. The residue was dried *in vacuo*, over concentrated H_2SO_4 and

Table II

Inhibitor Content and Dry Weight of Various Fractions of Silkworm Blood Plasma

Fractions	Total dry weight (g.)	Unit of inhibitor[1] per g. dry weight	Total units[1] of inhibitor
Blood plasma	*ca.* 150	33~80	5,000~12,000
Boiled extract	69.6	138	9,650
Silver precipitate	34.4	—	—
H_2S-treated supernatant	11.3	640	7,250
Non-adsorbable on Norit	8.6	835	7,180
Norit-adsorbed fraction (eluted with pyridine)	2.34	0	0
Amberite IR-120-effluent	4.69	*ca.* 1,500	*ca.* 7,000
Amberite IR-120-adsorbed (eluted with *N* NH_4OH)	2.62	147	385
Amberite IR-4B-effluent	0.204	27,100	5,540
Amberite IR-4B-adsorbed (eluted with *N* HCl)	2.55	404	1,030

1) Inhibitor units (definition in text) were calculated from the values of per cent inhibition obtained by incubating various amounts of each fractions (after adjustment of pH to 6.8) with crystalline cow liver catalase.

solid KOH. The dried material was redissolved in 100 ml. water and a 15 ml. portion was passed through an Amberite IR-120 column (1 cm.×10 cm., H^+-form). More than 90 per cent of the inhibitor was found to pass through the column. The effluent (pH 1.2) was then passed through an Amberite IR-4B column (1 cm.×10 cm., OH^--form), which was found to be ineffective in capturing the inhibiting principle, about 80 per cent of the activity being recovered in the effluent (pH 6.0).

The inhibitor content and the total dry weight of each fraction are summarized in Table II. The figures in this table indicate the values corresponding to 34.4 g. of the silver precipitate. It will be seen from these figures that the specific activity of the inhibitor was raised by about 500 times as compared with that of the original plasma; the inhibiting activity of the most highly purified preparation as measured according to the above prescription amounted to 27,100 units per g. dry weight.

Role of Nitrate and Nitrite in Catalase Inhibition—Table III shows the results of an experiment in which nitrate and nitrite were added to the reaction mixture together with the purified inhibitor (silver precipitate fraction). It will be seen that the inhibition is much greater than that calculated from the grades of inhibition of each substances added separately. It will also be seen that the augmenting effect of anions almost disappears when these concentrations are lowered to 1/10 of the concentrations in original blood plasma, and that chloride has the same effect when added in 0.02 *M*, and sulfate has no effect up to 0.01 *M*.

This effect of anions on the inhibition was also observed with more highly purified inhibitor. Fig. 1 shows the effect of nitrate concentration on the inhibitory action of an Amberite IR-4B-purified inhibitor. It will be seen that the inhibitory action of the inhibitor in question requires the presence of nitrate in the solution (no significant suppression of the reaction occurs at nitrate concentrations lower than 10^{-5} *M*, even in the

TABLE III

Effect of Nitrate and Other Inorganic Anions on Catalase[1] Inhibiting Activity of Silver Precipitate Fraction[2]

Silver precipitate fraction	Salts added[3]	Per cent inhibition
+	none	33.3
−	5×10^{-4} *M* KNO_3	11.5
+	,, ,,	76.9
−	5×10^{-5} *M* ,,	0
+	,, ,,	26.3
−	1×10^{-5} *M* $NaNO_2$	4.8
+	,, ,,	65.4
−	1×10^{-6} *M* ,,	0
+	,, ,,	35.7
−	5×10^{-4} *M* KCl	0
+	,, ,,	33.8
−	2×10^{-2} *M* KCl	6.8
+	,, ,,	64.5
−	1×10^{-2} *M* K_2SO_4	0
+	,, ,,	32.5

1) Cow liver catalase, recrystallized preparation.

2) Prepared from 50 ml. boiled extract of silkworm blood plasma, and reconstructed in the same volume of distilled water.

3) Indicates the final concentration in the incubation mixture.

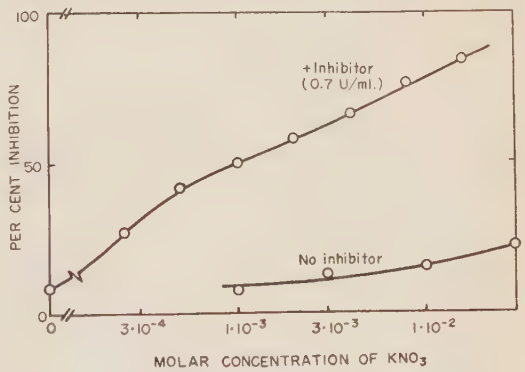

FIG. 1. Effect of nitrate on the catalase inhibiting activity of the purified inhibitor. Per cent inhibition was determined in the experimental conditions described in text. Concentration of the inhibitor (an effluent from Amberite IR-4B column) was 0.7 unit/ml. in incubation mixture.

presence of the purified inhibitor in the reaction mixture).

The effect of inhibitor concentration on the inhibition is shown in Fig. 2. The inhi-

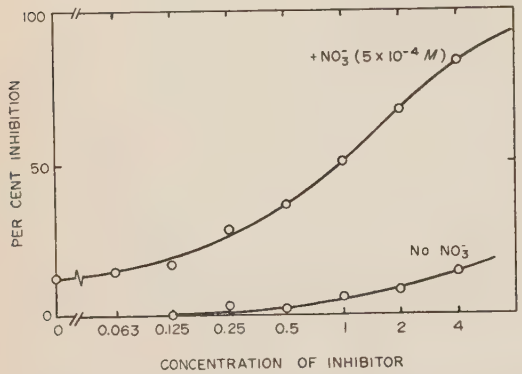

Fig. 2. Effect of inhibitor concentration on the inhibition of catalase. The abscissa represents the concentration (inhibitor units/ml.) of the purified inhibitor.

bition curve obtained is in good accord with the theoretical sigmoid curve drawn according to the formula

$$H=\frac{100}{1+\left(\frac{\Phi_I}{[I]}\right)^n}$$

where H, [I] and Φ_I represent per cent inhibition, inhibitor concentration, and the inhibitor concentration at 50 per cent inhibition, respectively, assuming n (the number of inhibitor molecules which combine with one enzyme molecule)=1 (*cf.* the preceding paper (*1*)).

Properties of the Purified Inhibitor—The purified inhibitor gives negative reaction to ninhydrin, nitroprusside and aniline hydrogen phthalate, and is weakly positive to ammoniacal silver nitrate. It gave also negative reaction to diphenylamine-sulfuric acid, showing the absence of nitrate in the preparation.

DISCUSSION

One of the most prominent features disclosed in this study is the mutually augmenting action of nitrate (or nitrite) and a natural inhibitor found in silkworm blood plasma, in suppressing catalase reaction. Even the concentrations of nitrate and nitrite actually occuring in the silkworm blood are sufficient to exhibit this synergetic effect. The above described finding that the dilution of silkworm blood plasma caused more or less significant increase in its catalatic activity (*1*), will be accounted for as an result of dilution of anion in the blood plasma. It will also be noted that other ions such as chloride can replace nitrate and nitrite (Table III) in this respect. Although actual concentration of chloride in silkworm blood (according to Portier and Duval (*6*), $8\sim21\times10^{-3}$ *M* per liter silkworm blood) is not so high enough to exert an effective influence on the process under investigation.

As to the nature of the inhibitor under investigation, or its mechanism of action, any definite conclusion will be premature, since no ultimate purification of the substance has yet been obtained.

The possibility was presented by Alexander (*7*) and Margoriash and Novogrodsky (*8*), who showed the mechanism of catalase inhibition by boiled aqueous extract of rat tissues to be due to the autoxidizable substances, which affect the catalase activity by generating hydrogen peroxide and forming an inactive catalase-H_2O_2 compound II. Regarding the autoxidizable nature of the silkworm inhibitor, it is conceivable that the same mechanism would be included in the catalase inhibition of silkworm blood plasma. In fact, slight increase of catalatic activity was observed when ethanol (*cf.* (*9*)) was added to blood plasma*. However, ascorbate and cysteine are excluded in the present case, at least judged from the non-adsorbability on the resin column. In addition, it will be noted that the inhibiting action of ascorbate or cysteine on crystalline catalase increases with increasing temperature and pH*, while the reverse is the case with the silkworm blood inhibitor (*1*).

The other possibility that the inhibitor may be amino acids (suggested by Ceriotti *et al.* (*10*)), which are fairly abundant in silkworm blood (*11*) was also excluded, since

* Ohoka, T.; unpublished data.

only 10 per cent of the inhibitory activity was retained on Amberite IR-120 column. It is, however, possible that amino acids might interact in some way with other inhibiting material in catalase inhibition by blood plasma.

SUMMARY

1. A catalase inhibitor was isolated from silkworm blood plasma in a partially purified form, its specific activity being raised to about 500-fold as compared with the starting material.

2. The inhibiting action of the inhibitor was found to be intensified by preincubating the enzyme in the presence of nitrate, nitrite or chloride.

3. The increase in catalatic activity, observed on dilution of silkworm blood plasma, was explained by this effect of the anions present in the blood plasma.

The author wishes to express his thanks to Prof. A. Takamiya of the Tokyo University for valuable advices on this work.

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Amide Metabolism in Yeasts

I. The Phenol-hypochlorite Method for the Estimation of Amides and Related Compounds in Uptake Studies

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(Received for publication, February 20, 1961)

A simple rapid assay method for measuring amide compounds was needed for uptake studies on yeast. Several procedures such as (a) ninhydrin, (b) diacetyl monoxime and (c) phenol-hypochlorite reactions were investigated. From a comparative study it appeared that the latter reaction had many possibilities and it will be described here *in extenso*.

Thomas (*1*, *2*) originally attributed the phenol-hypochlorite reaction (abbreviated PHC) to Berthelot, although as early as 1870 Lex (*3*) had observed that ammonia when heated in the presence of phenol and hypochlorite gave a green to blue color. Raschig (*4*), has investigated this reaction in some detail.

The PHC method has been used by many workers for the determination of ammonia. Orr (*5*) used NaOCl and phenol for routine analyses of ammonia in urine. Murray (*6*) applied this reaction to the estimation of ammonia derived from blood urea. Van Slyke and Hiller (*7*) first employed calcium hypochlorite solutions and Borsook (*8*) introduced modifications of this procedure. Russell (*9*) used Mn ion as a catalyst and Zalta and Lubochinky (*10*) replaced Mn by sodium nitroprusside. The PHC method has also been examined by Casas and Dominguez (*11*) and Pelaez and Konig (*12*) for ammonia determinations.

However, the PHC reaction is also applicable to substances other than ammonia. Thus, in a paper chromatographic investigation of substances in urine, Berry *et al.* (*13*) showed that allantoin, acetamide, urea and phenobarbital appeared as yellow to green spots on sheets that had been sprayed with phenol and hypochlorite. Preliminary investigations revealed that allantoin and urea did give green colors when treated with sodium hypochlorite solution (Clorox) and phenol, but the color was not very stable. In further experiments it was found that calcium hypochlorite solutions were more effective than Clorox, particularly if employed at a pH of 5 or lower. Upon addition of alkaline phenol, a deep blue color was obtained. Based upon these initial observations, a method was evolved for allantoin, which was subsequently found to be applicable to many amide or amide like compounds.

MATERIALS AND METHODS

Calcium hypochlorite, practical (*Union Chimique Belge*)—Five grams of the powder were suspended in 100 ml. H_2O, and the suspension was allowed to stand for 24 hours at room temperature with occasional stirring. At the end of this time the suspension was filtered, poured into small brown bottles and stored at 4°C.

Phenol—Chromatography grade phenol was saturated with water and the water saturated fraction mixed with methanol in the ratio of 1 : 1. The phenol solution was kept in a brown bottle and stored at 4°C. Prior to use 20 ml. were mixed with 5 ml. of 20% NaOH.

Potassium Phthalate Buffer, M/5, pH 4.20—This buffer was prepared according to Clark (*4*). Commercial phthalate was found to contain substances which increased the optical density of the blank.

Procedure—Into test tubes containing 10 to 50 μg. allantoin in 1 ml. H_2O is pipetted 1 ml. of the phtha-

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late buffer followed by 0.5 ml. of the hypochlorite reagent. After allowing to stand for twenty minutes at room temperature, 0.5 ml. of the alkaline phenol reagent is added down the side of the tube and the contents are shaken immediately. A blue color appears almost at once, and develops for at least 20 minutes. The contents of the tube are made to a suitable volume with water *i.e.* 6, or 10 ml., and the optical density is read at the wavelength of 625 mμ.

RESULTS AND DISCUSSION

Absorption Spectrum—An absorption maximum was found between 620 and 625 mμ (Fig. 1).

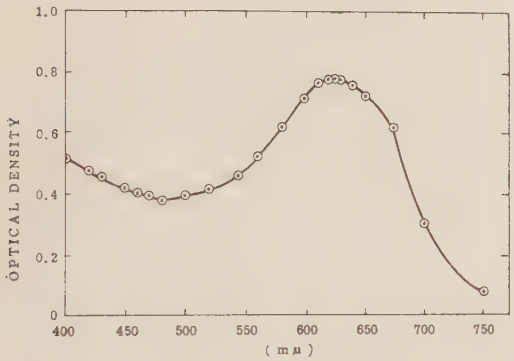

Fig. 1. Absorption spectrum of the dye formed in the allantoin phenol-hypochlorite reaction.

Effect of pH—Optimum color formation was obtained when the pH of the hypochlorite phase was maintained below pH 5 (Fig. 2). It was found necessary to use a buffer whose concentration was at least *M*/5; when

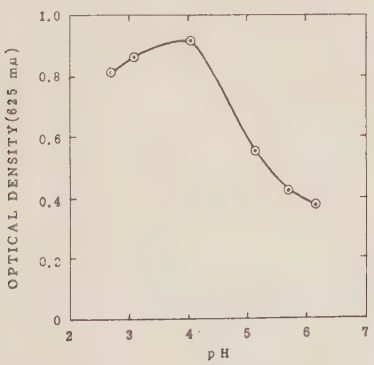

Fig. 2. Effect of variation of the pH of the hypochlorite phase on dye production in the allantoin phenol-hypochlorite reaction.

M/20 buffer was employed, the pH was found to vary considerably, and the colors obtained upon addition of alkaline phenol varied from light blue to green and yellow. The blue color appearing upon addition of alkaline phenol showed greatest intensity when the final pH was higher than 9.

Color Stability—It was found necessary to allow the hypochlorite to act for at least twenty minutes prior to addition of phenol. This period of time was found optimal for allantoin. After alkaline phenol addition, the color develops rapidly and reaches a maximum at the end of 15 minutes (Fig. 3). The color is stable for at least one hour at room temperature.

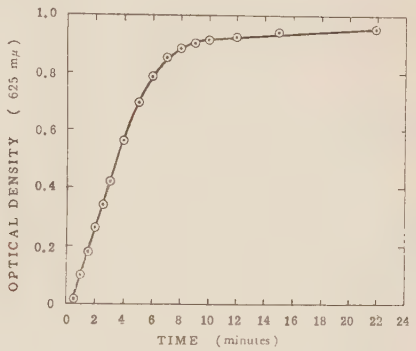

Fig. 3. Rate of formation of the dye produced in the allantoin phenol-hypochlorite reaction.

Order of Addition of Reagents—It was not possible to alter the order of addition of the reagents.

Beer's Law Relationship—The developed color follows Beer's law from 10 to 50 μg. of allantoin tested with an estimated error of $\pm 5\%$.

Specificity—Table I shows a list of compounds that were tested with the PHC reagents, using the procedure developed for allantoin. The absorption maximum of all compounds tested was found to be between 620 and 630 mμ. Although the color values of the amino acids are not very high, these substances may interfere in a determination if present in quantity. The most reactive amino acids seem to be those having short chain lengths such as glycine and alanine.

Table I

Compounds Tested with PHC Reagents

Color reactions of various compounds are compared using the PHC reagents and the procedure described. Values were expressed as a percentage of the absorbancy obtained with ammonium sulfate nitrogen.

Test Compound	Color value for test compound expressed as % $(NH_4)_2SO_4$ nitrogen
Ammonium sulfate nitrogen	100%
Glycine	8.9±1.1
Alanine	8.9±0.8
β-Alanine	<2
Serine	2.9±0.6
Threonine	2.3±0.8
Aspartic acid	6.0±0.5
Glutamic acid	<2
Taurine	<2
Cysteine-HCl	<2
Methionine	<2
Leucine	2.9±0.5
Isoleucine	2.6±0.6
Valine	2.9±0.2
Tyrosine	2.7±0.2
Phenylalanine	<2
Arginine	2.5±0.3
Tryptophan	<2
Histidine	<2
Hydroxyproline	<2
Acetamide	24.9±2.2
Propionamide	26.0±2.2
Asparagine	17.6±1.2
Urea	59.8±6.7
Allantoin	35.5±5.0
Nicotinamide	10.3±0.7
Creatine	16.3±1.8
Alloxan	20.6±2.7
Uracil	20.0±1.7
Guanine	52.3±8.2
Uric acid	19.2±2.1
Ethyl carbamate	5.5±0.5

Aspartic acid gave measurable color; β-alanine and glutamic acid practically none. Primary amides, such as acetamide and propionamide gave good color values with PHC reagents. The value obtained for asparagine, in contrast to aspartic acid, is comparable to those of acetamide and propionamide, indicating that the amide group is primarily involved in the reaction with PHC.

Most other substances tested are related to, or are derivatives of urea, and the latter compound gave the highest value, followed by guanine. Alloxan, uracil and uric acid all appear to have the same degree of reactivity with respect to phenol and hypochlorite. Nicotinamide reacted rather well, although more often than not this substance gave a green color, and repeated efforts were needed to obtain close replicates.

Tests in Biological Systems—Since the procedure was primarily designed for uptake investigations of amides in yeast suspensions, tests were run in the medium to be employed in order to observe interfering factors. Because the yeast *Candida utilis* was to be investigated the medium of Schultz and Atkin (*15*) was selected. For uptake studies of nitrogenous compounds, the following dilution are used, 5.26 ml. citrate buffer, 2.10 ml. salt solution, 5 g. glucose to a final volume of 100 ml. The ammonium sulfate nitrogen source is omitted in uptake studies.

The effect of the various components of the medium on the PHC reaction was examined by preparing standard curves of urea from solutions containing: citrate buffer only, citrate buffer and salts, citrate buffer, salts and glucose, glucose and salts, and glucose only. Corresponding control solutions were also prepared (without urea). One ml. quantities of control and test solutions were diluted to 10 ml. with water, and varying aliquots of the latter were taken for PHC analysis. The blank consisted of 1 ml. of the diluted control and the various urea aliquots were made to 1 ml. by appropriate serial additions of the diluted control. From this point on, the standard curve preparation was identical to the procedure described for allantoin. The results of this investigation are shown in Fig. 4.

It is evident that both citrate and glucose depress color formation considerably. Of the two interfering factors glucose appears to be

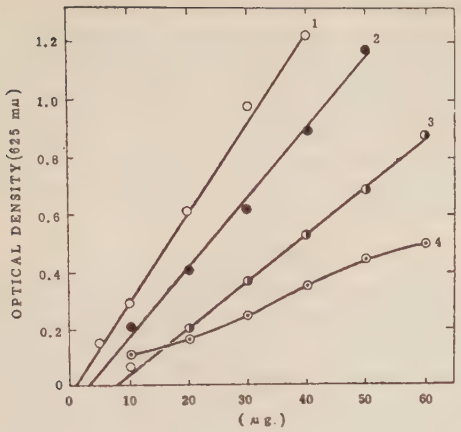

FIG. 4. Effect of various components of the Schultz and Atkins medium on dye formation by the urea phenol-hypochlorite reaction. Experimental conditions described in tests in biological systems. 1: urea in water, 2: urea in citrate buffer, 3: urea with all components of the Schultz and Atkins medium, 4: urea in 5% glucose solution.

the most important. Standard curves made from a combination of salts and glucose, and glucose and citrate (not shown) were identical to that of glucose alone. Salts apparently do not interfere with the determinations. Media containing all the components gave a good straight line with values considerably higher than those obtained from glucose alone. Although urea was used as the test substance, other compouds such as allantoin and acetamide exhibited similar behavior.

Following this phase of investigation, it was decided to test recovery of urea from the complete uptake media. One gram wet weight *Candida utilis* was suspended in 95 ml. of medium and 50 mg. of urea previously dissolved in 5 ml. H_2O added. One ml. samples were removed, pipetted into 9 ml. H_2O and centrifuged immediately. Procedures for the preparations of standard curves were the same as outlined above. Two conditions were investigated, (a) the suspension was aerated for one and a half hours prior to addition of substrate and (b) no aeration. The results of this study are shown in Fig. 5. It can be seen that a certain amount of the interfering factors (presumably glucose) is removed by aeration of the yeast suspension. It was also noted that the recovery figures varied depending on the precision with which the media was prepared, the quantity of yeast suspended and to a lesser extent on the age of the hypochlorite solution. The variation in the recovery was estimated as ±12%. Tests of yeast suspensions in the complete medium minus substrate, for interfering nitrogenous substances were negative.

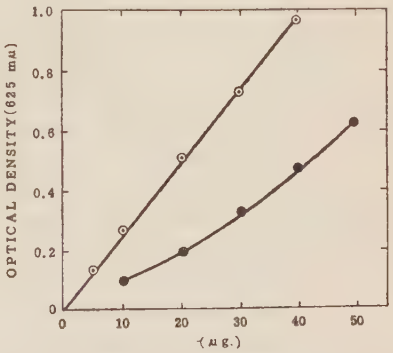

FIG. 5. Effect of aeration of the yeast suspension on dye production by the urea phenol-hypochlorite reaction. —⊙— suspension aerated one and a half hours prior to addition of urea. —●— no aeration.

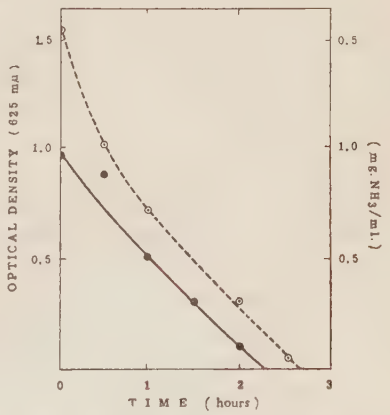

FIG. 6. Uptake of ammonia by cells of *Candida utilis*. —⊙— phenol-hypochlorite method, left-hand ordinate. —●— Ammonia determination by micro-distillation and nesslerization, right-hand ordinate. Initial ammonia concentration was 1 mg./ml.

In actual practice, blanks were prepared from dilutions of complete medium minus nitrogen source. Standard were prepared in the same manner using the substance to be investigated and the yeast was aerated for two hours prior to addition of the substrate. An example of the use of the PHC method is shown in Fig. 6 where uptake of ammonia by yeast cells is demonstrated. Ammonia was measured directly in yeast culture supernatants by use of the PHC method. Uptake of ammonia was cross checked by nesslerization of ammonia after micro-distillation with Conway diffusion vessels. It was noted that the PHC method was more sensitive, a finding in agreement with that of Thomas (*13*), Van Slyke and Hiller (*14*) and others (*9–15*). The PHC method has the added advantage of direct measurement. At the end of uptake, *i.e.* when no ammonia was left in the medium, PHC reaction of the medium gave a dull red color. This color appeared to be due primarily to undefined reducing substances in the medium and did not affect the determination of ammonia at the level employed. It was also observed that a certain amount of flocculation occurred in yeast medium supernatants upon addition of alkaline phenol. This flocculent material could be effectively dispersed by shaking, and usually did not reappear. In view of these findings, it is suggested that whenever uptakes of amide compounds are to be studied, that the effects of the medium on PHC reagents be investigated first.

Attempts were made to apply the ninhydrin* and diacetyl monoxime* methods to the estimation of allantoin and related compounds in uptake studies, but both procedures have serious drawbacks. While the former was found to be sensitive (10 to 100 μg. allantoin per ml.) it was found necessary to heat for 45 minutes. In addition ninhydrin reacts with amines, amino acids and may other nitrogenous substances. The diacetyl monoxime procedure also requires a heating step, and was found rather insensitive (at least 100 μg. allantoin per ml.). It was thus felt that despite some drawbacks in the PHC reaction, the method offered certain advantages, such as high reactivity with amides, ease of procedure and no heating steps. A report demonstrating the use of the PHC method in uptake experiments is in preparation.

* Domnas, A., unpublished results.

SUMMARY

A new method has been described for the colorimetric estimation of amide and amide-like compounds, based on the phenol-hypochlorite reaction. Various factors such as effect of pH, color stability, order of reagent addition, Beer's law relationship and specificity of reaction have been investigated. Water solutions, of allantoin, the main test compound can be determined in the range of 10–50 μg. with an estimated precision of ±5%. Procedures are described for using the phenol-hypochlorite method in uptake studies. Tests in biological systems show that glucose, citrate and unidentified reducing substances are interfering factors.

The author wishes to thank Prof. L. Massart in whose laboratories this work was completed, and Dr. J. De Ley for his kindly criticism and advice. The author is indebted to the Arthritis and Rheumatism Foundation, New York, for financial assistance which permitted this investigation.

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Comparative Biochemistry of Choline Sulfate Metabolism

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Choline sulfate, $(CH_3)_3N^+ \cdot CH_2 \cdot CH_2 \cdot O \cdot SO_3^-$, was first isolated by Woolly and Peterson (*1*) from mycelium of *Aspergillus sydowi*. Since then, its existence has been confirmed in many other fungi (*2–7*) as well as in a red alga (*8*) and in some of lichens (*9*).

In the preceding studies (*2*, *6*, *10–12*), it was reported that the production of choline sulfate was confirmed in several fungi belonging to Ascomycetes, Phycomycetes and Fungi Imperfecti, and also that the ester could be utilized as the sole source of sulfur for growth of these fungi.

Penicillium notatum and *Penicillium chrysogenum*, utilize choline sulfate as a sulfur source in their Penicillin synthesis (*4*, *16*, *17*). Takebe (*18*) showed that, a fairly large amount of the sulfate ester was contained in the extract of conidiospores of *Aspergillus niger*, from which it was considered to be a storage form of sulfate ion in the mold. It was stated by Harada *et al.* (*7*) that the production of choline sulfate in several fungi is due to the presence of choline sulfokinase. An extensive study of the role of choline sulfate in the sulfur metabolism of fungi has been undertaken by Spencer *et al.* (*21*) and they considered choline sulfate in fungal mycelia as a store of easily assimilated sulfate existing in an activated state. These results suggest that choline sulfate may play an important role in the sulfur metabolism of several fungi.

In the present investigation, experiments were carried out with a higher plant (*Vigna sesquipedalis*), a higher animal (mouse) and several microorganisms, with a view of studying the distribution of choline sulfate in nature.

Experimentals and Results

Experiment with Vigna sesquipedalis

Basal Medium—One liter of tap water containing; 20 g. glucose, 1 g. KNO_3, 0.6 g. $NaH_2PO_4 \cdot H_2O$, 0.2 g. $MgCl_2 \cdot 6H_2O$, 0.12 g. $CaCl_2 \cdot 2H_2O$ and 0.01 g. $FeCl_3 \cdot 6H_2O$.

Culture Medium—S^{35}-labeled inorganic sulfate (Medium 1) or S^{35}-labeled choline sulfate (Medium 2) was added to the basal medium as sulfur source (final concentrations, 0.001 *M*), and the pH values were adjusted to 5.8. The total radioactivity (S^{35}) in Medium 1 and 2 was *ca.* 120 μc per liter.

Ten ml. of each Medium (1 and 2) was accurately measured into conical flasks (30 ml.) plugged with cotton, and sterilized at 100°C for 30 minutes on three successive days. According to Kumada's method (*12*), dry seeds of *Vigna sesquipedalis* were sterilized by immersing them in a solution of hypochlorite of lime (content of active chlorine, 5,000 p.p.m.) for 90 minutes, washed several times with sterilized distilled water and placed on a wet filter paper in a aseptic Petri-dish. After incubation at 28°C for 2 days, were cut off about 15 mm. of a cuspated part of the roots that elongated about 30 mm. long after the initiation of growth of the embryo, transferred into the medium in flasks (two pieces of the excised roots per one flask) and incubated at 28°C for 5–7 days. The root pieces were washed three times with water (5 ml. at a time), made free from adherent water by putting them between filter paper sheets and weighed immediately.

As is seen in Table I, the root pieces in Medium 1 showed normal growth as such, *i.e.*, the tap-roots elongated to about 60–70 mm. in length and 5–10 lateral roots per one

TABLE I

Influence of Sulfur Source on Growth of Root Pieces

Medium	Incubation period (days)	Weight of 30 pieces of fresh roots (mg.)
M-1	5	2,740
M-1	7	3,221
M-2	5	1,842
M-2	7	1,985

root piece grew out. In Medium 2, on the contrary, most pieces showed remarkably poorer growth without bearing lateral roots, as compared with the control. Thirty pieces of roots of 7 days incubation were homogenized in a mortar, added with 30 ml. of water and transferred into a beaker. Then it was covered by a watch glass and heated for 20 minutes on a boiling water bath. After cooling, equi-volume of ethanol was added. The insoluble cell material was centrifuged off and the supernatant was concentrated up to about 2 ml. under reduced pressure. In the one-dimensional paper chromatography, the sample to be tested was placed upon a filter paper strip (Toyo No. 50), together with synthetic S^{35}-labeled choline sulfate (see below, experiment in mouse) for the purpose of comparison, and allowed to run overnigt in one of the following solvents: Solvent (I), an upper layer of *n*-butanol-acetic acid-water (5 : 1 : 4, v/v). Solvent (II), *n*-propanol-water (7 : 3, v/v). The obtained chromatogram was cut into segments of 20 mm. long. The radioactivity in each segment was measured with a Geiger-Müller couter. All the radioactivity values in the present paper were corrected for the back ground counts.

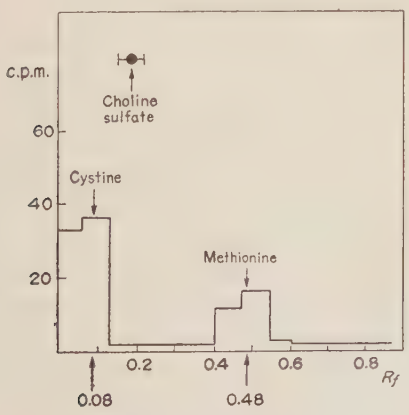

FIG. 1. Chromatogram with Solvent (I).

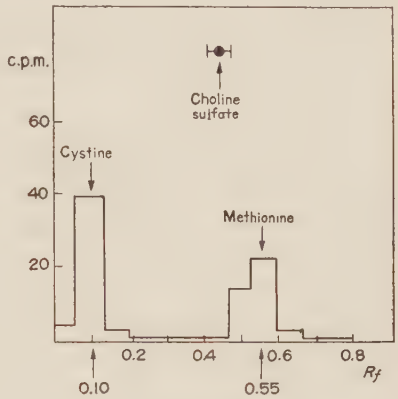

FIG. 2. Chromatogram with Solvent (II).

Figs. 1 and 2 indicate the chromatogram of hot-water extracts from 30 pieces of roots incubated for 7 days in the Medium 1. The location of the spot for S^{35}-labeled choline sulfate is indicated at the top of the graph for the purpose of comparison. The R_f-values of the organic ester are 0.18 in Solvent (I) and 0.42 in Solvent (II), respectively. In each chromatogram of the hot water extracts, there appeared two peaks of radioactivity, the positions of which were marked as 0.08 and 0.48 in Solvent (I), and, 0.10 and 0.55 in Solvent (II) in R_f-values, respectively. These values were well coincided with those of cystine and methionine which were chromatographed using the same Solvents (I) and (II). From these findings, it is reasonably concluded that, when inorganic sulfate is given as sulfur source to the plant, no choline sulfate is formed as an intermediate of sulfur metabolism. The same result was obtained for the samples incubated for 5 days. In the Medium 2, the root pieces showed only poor growth, as described above, and any radioactivity has been found out neither in the hot-water extracts nor in the insoluble cell material that were prepared after the same method as above.

From these experimental results, the conclusion that the organism does not utilize choline sulfate as sulfur source, should be drawn.

Experiment with Mouse

S^{35}-labeled choline sulfate was prepared from S^{35}-labeled sulfuric acid (1 mc) and choline chloride by the method of Schmidt and Wagner (*13*). The specific radioactivity of the recrystallized sample was 1,100 c.p.m. per mg.

Mice were bred for a week prior to their treatment in order to select individuals of homogeneous physical conditions. Among a lot of these mice nine weighing about 20 g. were selected, and they were divided into three groups (three members in each group, numbered 1–3). 0.1 ml. of an aqueous solution of S^{35}-labeled choline sulfate (0.1 *M*) was injected subcutaneously for each mouse. The quantity of choline sulfate injected was 1.83 mg. per one mouse. Mice of the respective groups were sacrificed 5-, 12-, and 24-hours after the injection. The blood was sucked out from hearts by means of a syringe and, three kinds of viscera, the livers, kidneys and brains were nipped off as soon as possible. The radioactivities of each of these organs from three individuals of the same group and those of urine gathered from each group were measured.

1. Radioactivity Measurements in Livers, Kidneys, Brains and Blood—The average wet weight of organs of each kind was *ca.* 1000 mg. for a liver, *ca.* 250 mg. for a pair of kidneys and *ca.* 350 mg. for a brain, respectively. Respective organs obtained from three mice were homogenized at a time with five volumes of dislilled water in a Potter-Elvehjem homogenizer. The samples free from proteins and salts were prepared according to Crammer's method (*15*), dried on dishes using infrared lamp, and their radioactivities were measured.

The average quantity of blood obtainable from one heart was *ca.* 0.25 ml. Five ml. of distilled water was added to 0.75 ml. of blood (obtained from three mice), which was treated by the same method as above. As is seen in Table II, traces of radioactivity were observed only in livers and blood of mice belonging to the first group which were sacrified 5 hours after the injection. In other groups, the results were negligible or perfectly negative.

TABLE II

Radioactivity Distribution in Livers, Kidneys, Brains and Blood

Group	Total Radioactivity (c. p. m.)			
	Liver	Kidney	Brain	Blood
1	24(0.4%)	11	13	22(0.36%)
2	15	0	0	12
3	0	0	0	0

2. Radioactive Substance Found in Urine—Urine was gathered by the group. Five ml. of water was added to them. They were treated by the same proceure as in the case of tissue and their radioactivities were measured. As shown in Table III, about 93 per cent of the injected S^{35} was found in urine for the first group, in which mice were sacrificed 5 hours after the injection.

TABLE III

S^{35} Found in Urine

Group	Total Radioactivity (c. p. m.)
1	5,590 (92.7%)
2	5,620 (93.3%)
3	5,612 (93.0%)

In order to identify this radioactive substance, the samples prepared by treating urine after Crammer's method, were concentrated and chromatographed by the same method as described for the hot-water extracts of plant roots. In each chromatogram there appeared a single peak of radioactivity, corresponding to R_f-values of 0.18 in Solvent (I), 0.42 in Solvent (II) which are ascribed to choline sulfate.

Experiments with Microorganisms

The synthetic media used are as follows:

Stock Solution—Basal Medium-1—(BM-1): One liter containing; 100 g. glycerol, 10 g. glucose, 1.4 g. asparagine, 1.8 g. K_2HPO_4, 0.9 g. sodium citrate and 0.3 g. ferric citrate (pH 7.2).

Basal Medium-2 (BM-2): One liter containing; 10 g. glucose, 4 g. $(NH_4)_2HPO_4$, 5 g. NaCl, 2 g. K_2HPO_4, 0.4 g. $CaCl_2 \cdot 2H_2O$ and 0.02 g. $FeCl_3 \cdot 6H_2O$ (pH 7.2).

Choline Sulfate Solution: One liter contains 18.3 g. of choline sulfate (0.1 *M*) and 20.3 g. of $MgCl_2 \cdot 6H_2O$ (0.1 *M*).

Magnesium Sulfate Solution: One liter contains 24.6 g. of $MgSO_4 \cdot 7H_2O$ (0.1 *M*).

Culture Medium—Each medium was prepared by mixing the above-mentioned stock solutions in the following proportions:

Medium-1'-CS (M-1'-CS):
BM-1 .....95 ml.
Choline sulfate solution... 5 ml. (0.005*M*)

Medium-1'-SO_4 (M-1'-SO_4):
BM-1 .....95 ml.
Magnesium sulfate solution .....5 ml. (0.005*M*)

Medium-2'-CS (M-2'-CS):
BM-2 .....95 ml.
Choline sulfate solution... 5 ml. (0.005*M*)

Medium-2'-SO_4 (M-2'-SO_4):
BM-2 .....95 ml.
Magnesium sulfate solution .....5 ml. (0.005*M*)

The medium (50 ml.) was accurately measured into a series of 300 ml. conical flasks plugged with cotton, and sterilized by heating at 100°C for 30 minutes on three successive days. Each flask containing 50 ml. of M-1'-CS (or M-1'-SO_4) with a sulfate content equivalent to 8 mg. sulfur/flask, was inoculated with a loopful of *Mycobacterium phlei* taken from culture grown on glycerol-nutrient-agar slant. The flasks were put on a shaker in the incubator at 28°C for 7 days. Bacterial cells were harvested and the quantity of choline sulfate (or inorganic sulfate) in the residual culture medium was determined by the same method as in the preceding paper (*10*) (Table IV).

As is apparent in Table IV, *Mycobacterium phlei* and *Streptomyces griseus* absorbed about 20 per cent of choline sulfate given as a sole source of sulfur but less favorable growth was shown in this medium with choline sulfate than in normal medium with inorganic

Table IV

Utilization of Choline Sulfate or Inorganic Sulfate as Sulfur Source for Growth of Bacteria

Organism[2]	Dry weight of bacterial cell (mg.)	Incubation period (days)	Medium	Utilization of choline sulfate[1]		Utilization of iroganic sulfate[1]	
				Sulfate utilized $BaSO_4$ mg./flask	%	Sulfate utilized $BaSO_4$ mg./flask	%
Mycobacterium phlei IFO 3158	204	7	M-1'-CS	12.3	21.1	—	—
	249	7	M-1'-SO_4	—	—	10.4	17.8
Streptomyces griseus IFO 3430	168	5	M-2'-CS	10.7	18.3	—	—
	127	5	M-2'-SO_4	—	—	11.0	18.9
Streptomyces antibioticus IFO 3126	35	5	M-2'-CS	1.0	negligible	—	—
	66	5	M-2'-SO_4	—	—	3.4	5.8

1) Initial amount of choline sulfate (or inorganic sulfate) in each flask; 58.2 mg. (On $BaSO_4$ basis).

2) IFO=Institute for Fermentation, Osaka.

sulfate. *Streptomyces antibioticus* failed to grow even in normal medium.

S^{35}-labeled inorganic sulfate (final-concentration 0.005 *M*) was added to the basal media, BM-1 and BM-2. The total radioactivity (S^{35}) of these media was about 30 μc per liter. *Mycobacterium phlei* and *Streptomyces griseus* were inoculated respectively in M-1'-$S^{35}O_4$ and M-2'-$S^{35}O_4$, and incubated at 28°C for 5–7 days. The presence of choline sulfate in the bacterial extracts was proved to be negative by the same method as in the previous paper (*6*).

Twenty-three fungi have been tested for the production of choline sulfate after the same method as in the previous paper (*6*) and the obtained results are included in Table V.

DISCUSSION

From the experimental results obtained in this study, it is apparent that choline sulfate is not accumulated in a detectable amount in the tissue of a higher plant, and, moreover, it is not utilized as sulfur source. As the ester was shown, in another experiment, to reveal no inhibiting action on the growth of the plant organ, we can arrive at the conclusion that choline sulfate is a completely indifferent material for the plant tissue so far tested. On the other hand, in the case of mouse, the subcutaneously injected ester is immediately recovered in urine, *i.e.*, even 5 hours after the injection, *ca.* 93 per cent of the given choline sulfate was excreted in urine. In blood and livers, only 0.36–0.40 per cent of the injected ester was found. Since about 25 per cent of total blood can be obtained from a heart which is nearly equal to the quantity in a liver, the remaining 50 per cent of blood is considered to be contained in other viscera and in the muscle. Accordingly, injected choline sulfate is considered to be immediately transferred into blood stream and rapidly excreted into urine in its original ester form without accumulating in a liver and other organs. It may be therefore concluded that this organic ester is completely indifferent material for the animal as well as for the plant.

It may be of some interest that *Mycobacterium phlei* and *Streptomyces griseus* absorbed 20 per cent of choline sulfate given as a sole sulfur source, despite the fact that they produced no choline sulfate from inorganic sulfate. In the preceding papers (*5*, *10*), the author reported the distribution of choline sulfate in fungi together with the fact that several fungi utilized choline sulfate as sulfur source. The micro-organisms studied by the author amounts to 42 strains, including the results of the present study. Similar investigations have been performed by Ballio *et al.* (*5*) and Harada *et al.* (*7*). In Table V, the results obtained in a survey of the occurrence of choline sulfate in various fungi are summarized together with those reported by the above-mentioned authors.

The taxonomic division of 93 fungi belonging to Phycomycetes, Ascomycetes, Basidiomycetes and Fungi Imperfecti is that adopted by Bessey (*19*). In the Phycomycetes, 16 strains except *Rhizopus oryzae* showed no production of choline sulfate. In the Protoascomycetes, 15 strains are choline sulfate negative with five exceptions (*Hansenula anomala*, *Rhodotorula rubra*, *Taphrina communis*, *Sporobolomyces roseum* and *Sp. salmonicola*) whereas in the Euascomycetes, 36 strains studied are all positive of choline sulfate production. In the Basidiomycetes, only *Ustilago scitaminea* belonging to the Teliosporeae was shown to be positive while 5 species belonging to Eubasidiae were all negative. In bacteria, the presence of choline sulfate has never been known. It appears therefore that the presence of choline sulfate is practically restricted to the members of the Euascomycetes and of the Fungi Imperfect, and it is negative, with only few exceptions, for those of Phycomycetes, Protoascomycetes, Basidiomycetes and Bacteria.

In 16 strains belonging to the two genera, *Aspergillus* and *Penicillium*, choline sulfate is consistently present. These species may be sought in the Fungi Imperfecti (Harada *et al.* and Ballio *et al.*, *loc. cit.*) but the present author has classified them as Aspergillales of

TABLE V

Occurrence of Choline Sulfate in Various Fungi

Taxonomic Position		Organism	Authors[3)]	Presence of Choline Sulfate
Order	Family			
Class: Phycomycetes				
Peronosporales	Pythiaceae	*Phytophthora infestans* IFO 4872	I+	−
〃	〃	*Ph.* sp.	B	−
Blastocladiales	Blastocladiaceae	*Allomyces macrogynus*	B	−
Mucorales	Mucoraceae	*Mucor mucedo* (−) IFO 5776	I+	−
〃	〃	*M. javanicus* (+) IFO 4569	I+	−
〃	〃	*M. hiemalis* (−)	H	−
〃	〃	*M.* sp.	B	−
〃	〃	*Rhizopus nigricans* IFO 5411	I	−
〃	〃	*R. nigricans*	B	−
〃	〃	*R. oryzae* IFO 4746	I	+
〃	〃	*R. stolonifer* (+)	H	−
〃	〃	*R. stolonifer* (−)	H	−
〃	〃	*Absidia glauca* IFO 4002	I+	−
〃	〃	*Zygorhynchus moelleri* IFO 5305	I+	−
〃	〃	*Phycomyces nitens* IFO 5694	I+	−
〃	〃	*Ph. blakesleeanus*	H	−
〃	Choanephoraceae	*Gunninghamella echinulata*	H	−
Class: Ascomycetes Subclass: Protoascomycetes				
Saccharomycetales	Saccharomycetaceae	*Saccharomyces cerevisiae* IFO 0209	I+	−
〃	〃	*S. cerevisiae*	H	−
〃	〃	*S. cerevisiae var. ellipsoideus*	H	−
〃	〃	*S.* Strain XII IFO 2113	I	−
〃	〃	*Pichia membranaefaciens*	H	−
〃	〃	*Hansenula anomala* IFO 4540	I	+
〃	〃	*Zygosaccharomyces marxianus* IFO 0219	I+	−
〃	〃	*Nematospora gossypii*	H	−
〃	〃	*Ashbya gossypii*	B	−
〃	〃	*Eremothecium ashbyii*	H	−
〃	〃	*E. ashbyii*	B	−
〃	Schizosaccharomycetaceae	*Schizosaccharomyces octosporus* IFO 0353	I+	−
〃	〃	*Dipodascus uninucleatus*	B	−
〃	Torulopsidaceae	*Torulopsis utilis*	B	−
〃	〃	*T. utilis*	H	−
〃	〃	*Candida tropicalis* var. *japonica* IFO 0618	I+	−
〃	Rhodotorulaceae	*Rhodotorula rubra*	H	+
〃	Sporobolomycetaceae	*Sporobolomyces roseum*	H	+
〃	〃	*Sp. salmonicolar*	H	+

Taxonomic Position		Organism	Authors[3]	Presence of Choline Sulfate
Order	Family			
Taphrinales	Taphrinaceae	*Taphrina communis*	B	+
Subclass: Euascomycetes				
Aspergillales (=Plectascales)	Aspergillaceae	*Aspergillus niger* IFO 4407	I	+
//	//	*Asp. niger*	B	+
//	//	*Asp. sydowi* IFO 4402	I	+
//	//	*Asp. sydowi*	H	+
//	//	*Asp. oryzae*[1]	I	+
//	//	*Asp. oryzae*	H	+
//	//	*Asp. foetidus* IFO 4031	I+	+
//	//	*Asp. terreus*	H	+
//	//	*Asp. nidulans*	H	+
//	//	*Penicillium notatum* IFO 4640	I	+
//	//	*P. notatum*	H	+
//	//	*P. chrysogenum* IFO 4626	I	+
//	//	*P. chrysogenum*	H	+
//	//	*P. chrysogenum*	B	+
//	//	*P. citreo-roseum*	H	+
//	//	*P. aurantio-brunneum*	H	+
//	//	*Monascus purpureus* IFO 4513	I	+
Sphaeriales	Melanosporaceae	*Neurospora crassa* IFO 6067	I+	+
//	//	*N. crassa* IFO 6178	I+	+
//	//	*N. crassa*	H	+
//	//	*N. crassa*	H	+
//	//	*N. crassa*	B	+
//	//	*N. sitophila* IFO 6070	I	+
//	//	*N. sitophila* (+)	H	+
//	//	*N. sitophila* (−)	H	+
//	//	*Melanospora* sp.	B	+
//	Ophiostomataceae	*Ceratostomella ulmi*	B	+
//	Allantosphaeriaceae	*Diatrype bullata*	B	+
//	Diaporthaceae	*Valsa ceratophora*	B	+
//	Xylariaceae	*Xylaria* sp.	B	+
Hypocreales	Nectriaceae	*Gibberella zeae*	B	+
//	Clavicipitaceae	*Claviceps purpurea* IFO 5782	I+	+
//	//	*Cl. purpurea*	B	+
Pseudosphaeriales	Pseudosphaeriaceae	*Pyrenophora calvescens* IFO 4865	I+	+
//	Botryosphaeriaceae	*Botryosphaeria guercum*	B	+
Pezizales	Sclerotiniaceae	*Sclerotinia* sp.	B	+
Class: Fungi Imperfecti			I	+
Moniliales	Moniliaceae	*Oospora lactis* IFO 4597		
//	//	*Botrytis cinerea* IFO 5964	I	+
//	//	*Verticillium dahliae* IFO 6150	I+	+

Taxonomic Position		Organism	Authors[3]	Presence of Choline Sulfate
Order	Family			
Moniliales	Moniliaceae	*Trichothecium roseum* IFO 6157	I	+
//	//	*Cephalosporium acremomium*	B	+
//	//	*Oedocephalum glomerulosum*	H	+
//	Dematiaceae	*Cladosporium herbarum* IFO 4458	I+	+
//	//	*Cl. cladosporioides*	H	+
//	//	*Pullularia pullulans* IFO 4464	I	+
//	//	*Alternaria tenuis* IFO 4026	I	+
//	//	*Alt. japonica* IFO 5244	I+	+
//	Tuberculariaceae	*Fusarium solani* IFO 5893	I	+
//	//	*F.* sp.	H	+
//	//	*F.* sp.	B	+
Class: Basidiomycetes				
Subclass: Teliosporeae				
Ustilaginales	Ustilaginaceae	*Ustilago scitaminea*	H	+
Subclass: Eubasidiae				
Polyporales	Polyporaceae	*Polystictus hirsutus*[2]	I+	−
//	//	*P. sanguineus*[2]	I+	−
//	//	*Fomes fomentarius*[2]	I+	−
Agaricales	Agaricaceae	*Schizophyllum commune*[2]	I+	−
//	//	*Pleurotus japonicus* Kawam.[2]	I+	−
Bacteria				
		Streptomyces griseus IFO 3430	I+	−
		Strept. albus sp.	H	−
		Mycobacterium phlei IFO 3158	I+	−
		Pseudomonas pyocyanus	H	−
		P. fluorescens	H	−
		Escherichia coli	H	−
		Aerobacter aerogenes	H	−
		A. aerogenes	H	−
		Staphylococcus aureus	H	−
		Streptococcus faecalis	H	−
		Bacillus subtilis	H	−

1) Obtained from the Faculty of Science, Nagoya University.

2) Obtained from the Faculty of Agriculture, Gifu University.

3) H=Harada, T. *et al.* (7)
B=Ballio, A. *et al.* (5)
I=Itahashi, M. (6)
I+=Itahashi, M. (present study)

the Euascomycetes because of the fact that some species belonging to these genera have perfect stages. The sulfate ester is present in the members (14 strains) of Fungi Imperfecti group, among which Moniliaceae (6 strains) and Dematiaceae (5 strains) are considered to be taxonomically related to Aspergillales since they have conidial stages similar to that of Aspergillales. On the other hand, macroconidial stages of *Fusarium* (3 strains) belonging to Tuberculariaceae apparently corresponds to the imperfect stages of *Gibberella zeae* and of various other Hypocreales, subclass Euascomycetes. Accordingly, these 14 strains of the Fungi Imperfecti are considered to have connections with species belonging to the subclass Euascomycetes. *Taphrina*, being morphologically considered to be a much simplified offshoot of the Pezizales, subclass Euascomycetes, is one of the few species belonging to Protoascomycetes in which, unusually choline sulfate production was observed. No experiment with Basidiomycetes was performed by Ballio *et al.* Of Basidiomycetes, as mentioned above, only *Ustilago scitaminea* was confirmed to produce choline sulfate. However, according to Linder (*20*), Uredinales and Ustilaginales are derived from Ascomycetes and are in the vicinity of Sphaeriales or Dothideales. From this view-point, *Ustilago* is considered to be in a connection with Euascomycetes. Of 5 strains belonging to Eubasidiae, none of them showed choline sulfate production. From these considerations, though very few exceptions still exist, it should be reasonablly concluded that the presence of choline sulfate is restricted to the fungi belonging to the Euascomycetes and other several fungi which are taxonomically closely related to the subclass. This fact appears to indicate an existence of a close relation between the distribution of the ester in fungi and phylogenic classification.

Referring to the fact that certain amounts of sulfate ion were liberated in the residual culture medium when choline sulfate was given to *Alternaria tenuis* (IFO 4026) as the sole sulfur source, it was stated, in the previous paper, that this strain may contain an enzyme capable of hydrolyzing the sulfate ester. However, in the later experiment performed with *Alternaria tenuis*, *Alt. japonica* (IFO 5244), and *Alt. porri* (IFO 5924), in any of these strains, such an enzyme has never been found out. Thus it should be reasonablly concluded that the sulfate ions liberated in the medium are those discharged as a final product in sulfur metabolism relative to the incorporation of choline sulfate.

SUMMARY

The presence of choline sulfate in a higher plant and the behavior of the exogenously supplied ester in the mouse were examined. As for the plant studied, the compound is neither formed in the tissue nor utilized as sulfur source. Choline sulfate injected into mouse bodies was very rapidly excreted into urine without changing its original form. The obtained results strongly suggest that this ester may be completely indifferent material both for the higher plant and for the animal.

The distribution of choline sulfate among 104 strains belonging to Phycomycetes, Ascomycetes, Fungi Imperfecti, Basidiomycetes and Bacteria has been summarized. The results so far obtained suggest that the presence of choline sulfate is restricted to the fungi belonging to the subclass Euascomycetes and other several fungi taxonomically closely relating to the subclass.

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Ribonuclease of *Streptomyces erythreus*

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In the studies of the structure of the biological macromolecules, much important information has been derived from the investigations of degradation products by highly specific enzymes.

Recently, Sato and Egami discovered two thermostable ribonucleases (T_1 and T_2) in an extract of *Aspergillus oryzae* (*1*). RNase T_1 was highly purified and the specificity of the enzyme was studied in detail (*1*, *2*).

This paper deals with the partial purification of the RNase of *Streptomyces erythreus* (NRRL 2338) and the study of the properties of the enzyme. The results of the study showed that the specificity of the enzyme is similar to that of RNase T_1.

METHODS

Materials

Yeast RNA—Commercial yeast RNA (Kirin Research Institute) was thoroughly dialyzed against distilled water and lyophilized.

Rabbit Appendix DNA—This was kindly given by Dr. T. Sakaki, Nagoya University. The sample was prepared from rabbit appendix by the method of Key, Simmons and Dounce (*3*).

Acrinol (*2-ethoxy-6, 9-diaminoacridinium lactate*)—Commercial preparation was used.

DEAE-cellulose—This was prepared by making use of Solka Floc SW-40 cellulose powder according to Peterson and Sober (*4*).

Cyclic Mononucleotides—These compounds were prepared by the liquid ammonia degradation of yeast RNA (*5*).

Pancreatic Ribonuclease "Core" of Yeast RNA—The "core" (RM-3) was prepared as in a previous report (*6*).

Prostatic PMase—This was prepared as in the previous report (*7*).

Measurement of Enzyme Activities

RNase—0.5 ml. of the enzyme solution in 0.2 *M* phosphate buffer (pH 7.4) was mixed with 0.5 ml. of RNA solution (6 mg./ml.). After incubating at 37°C for 15 minutes the reaction was stopped by addition of 0.2 ml. of 0.75% uranyl acetate in 25% perchloric acid. After standing for 20 minutes the precipitate was centrifuged off. 0.2 ml. of the supernatant fluid was diluted with distilled water to 5.0 ml. and optical density at 260 mμ was read. With each set of assays a control was run mixing the enzyme solution after introduction of the uranyl reagent. The blank value was substracted from the experimental readings. It was desirable to restrict the amount of enzyme so that the net optical density of the final diluted sample did not exceed 0.2.

Unit of RNase—One unit of RNase is defined as the amounts which cause a change of optical density ($\Delta O.D._{260}$) of 0.1 in the test as carried out above and specific activity was defined as units per unit absorbancy of the enzyme solution at 280 mμ with 1 cm. light path.

DNase—Method used was essentially the same as RNase activity measurement except that DNA solution (2 mg./ml.) was used as a substrate.

Phosphodiesterase—0.5 ml. of potassium diphenyl phosphate solution (2 mg./ml.) was added to the mixture of 0.5 ml. of enzyme solution and 0.5 ml. of 10^{-2} *M* $MgSO_4$ solution. After incubating at 37°C for one hour, 1.5 ml. of 0.4 *M* trichloroacetic acid was added to the reaction mixture and allowed to stand for 30 minutes in the incubator to complete precipitation. After removing the precipitate, the liberated phenol was measured by Hagihara's method (*8*).

PMase—0.5 ml. of enzyme solution and 0.5 ml. of

Abbreviations: ribonuclease, RNase; ribonucleic acid, RNA; phosphomonoesterase, PMase; desoxyribonuclease, DNase; desoxyribonucleic acid, DNA; guanosine-2', 3'-cyclic phosphate, G_p!; 2'-guanylic acid, 2'-G_p; 3'-guanylic acid, 3'-G_p; adenosine-2', 3'-cyclic phosphate, A_p!; 2'-adenylic acid, 2'-A_p; pyrimidine nucleotide, $(Py)_p$; pyrimidine nucleoside-2', 3'-cyclic phosphate, $(Py)_p$!.

10^{-2} *M* $MgSO_4$ solution were mixed with 0.5 ml. of sodium β-glycerophosphate (3 mg./ml.) and incubated at 37°C for one hour, and the reaction was stopped by adding 1.5 ml. of 0.4 *M* trichloroacetic acid, and the inorganic phosphate thus produced was determined by Allen's method (*9*).

Paper Chromatography

Single dimentional descending paper chromatography was carried out with Toyoroshi No. 51A paper. The following solvent systems were used: Solvent 1, saturated $(NH_4)_2SO_4$—*M* sodium acetate-*iso*propanol (80 : 18 : 2, v/v/v) (*10*). Solvent 2, *iso*propanol-water (70 : 30, v/v), with NH_3 in the vapor phase (*10*). Solvent 3, *tirt*-butanol, 70 ml., constant boiling HCl 13.2 ml., water to 100 ml. (*11*). The spots of nucleotide and nucleoside were visualized by means of germicidal lamp equipped with a Kaken ultraviolet filter or mineralight lamp. Spots of guanine and its derivatives could be easily distinguished from other spots, as they gave the fluorescent spots at acid pH. The prints were made by making use of Mitsubishi CH photographic paper. Spots of nucletides were differentiated from nonphosphorylated compounds by positive color test for organic phosphorus with Hans-Isherwood reagent (*12*).

Determination of Base Ratio

This was carried out by the paper chromatographic method of Smith and Markham with solvent 3 (*11*).

RESULTS

Purification of the Enzyme

Step 1. Precipitation with Acrinol—The culture broth of *Streptomyces erythreus* was clarified by filtration and erythromycine was extracted by organic solvents (*13*). Aqueous phase (residual broth) then was used as a source of the enzyme.

Seven and a half liters of the residual broth (pH 7.3) were diluted with 22.5 liters of water, followed by the addition of 2.4 liters of 1% aqueous acrinol solution under stirring and allowed to stand for 2 days in the cold room. The yellow precipitate produced was collected by centrifugation, and extracted with 500 ml. of *M* sodium acetate (pH 6.0) under stirring. The insoluble residue was extracted again with 300 ml. of the same buffer. To the combined extract 2 volumes

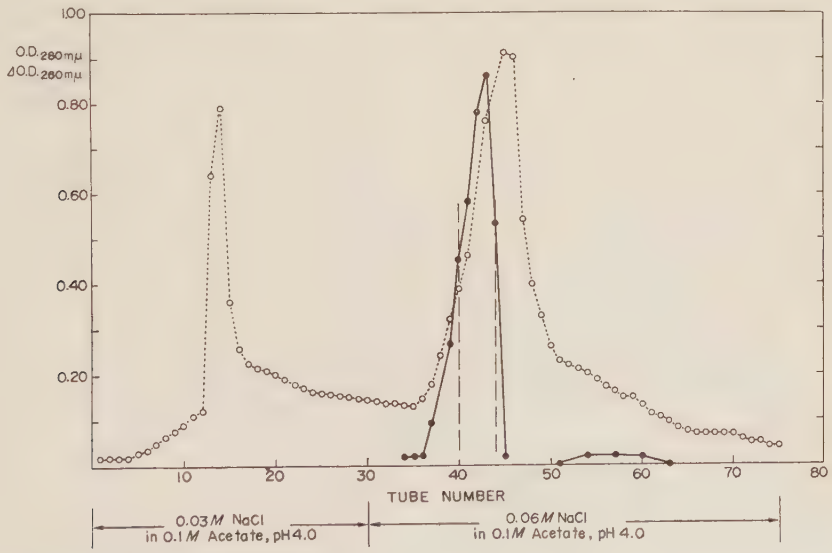

Fig. 1. Chromatography of RNase of *Streptomyces erythreus*.
--○-- Protein concentration (O. D. at 280 mμ)
—●— RNase activity (ΔO. D. at 260 mμ)
Exchanger, 5 g. of DEAE-cellulose; Column size, 14 cm. × 3.1 cm^2; Sorbed material, active effluent of previous DEAE-cellulose treatment from 8 g. of crude enzyme preparation; Fraction volume *ca.* 9.1 ml./tube. The activity was determined at protein concentration of O. D. 280 mμ=0.001 and calculated for original absorbancy of each of the fractions.

of cold acetone were added. After standing for 2 hours, the precipitate of crude RNase was thoroughly washed with cold acetone and dried under reduced pressure. All these operations were carried out in a cold room.

By the procedure described above, about 20 times increase in specific activity was obtained and a yield of RNase activity was about 34 per cent.

Step 2. Adsorption on DEAE-cellulose—Four grams of the crude preparation were dissolved in 100 ml. of 0.1 *M* acetate buffer (pH 4.0) and adsorbed on 10 g. of bufferized (pH 4.0) DEAE-cellulose on the glass filter. After washing with 300 ml. of the same buffer and 200 ml. of bufferized 0.02 *M* NaCl solution (0.1 *M* acetate, pH 4.0), an elution was carried out with 500 ml. of 0.06 *M* NaCl in 0.1 *M* acetate buffer (pH 4.0).

At this stage the enzyme was purified about 200-fold (activity yield 20 per cent). No phosphodiesterase activity and phosphomonoesterase activity were detected.

Step 3. Chromatography of DEAE-cellulose Column—Further purification was performed by DEAE-cellulose column chromatography. The active effluents obtained from first DEAE-cellulose treatment (from 8 g. crude preparation) were pooled, concentrated under reduced pressure and dialyzed against distilled water. The dialyzed solution was mixed with equal volume of 0.2 *M* acetate buffer (pH 4.0) and charged on 5 g. of bufferized DEAE-cellulose column (pH 4.0). Elution was carried out at pH 4.0 (0.1 *M* acetate) with stepwise increments in NaCl concentration (*cf.* Fig. 1).

Final purification was carried out by rechromatography on DEAE-cellulose column. The fractions comprising the main peak in Fig. 1 was pooled, dialyzed against distilled water and equal volume of 0.2 *M* acetate (pH 4.0) was added. The solution was submitted to a gradient elution on the

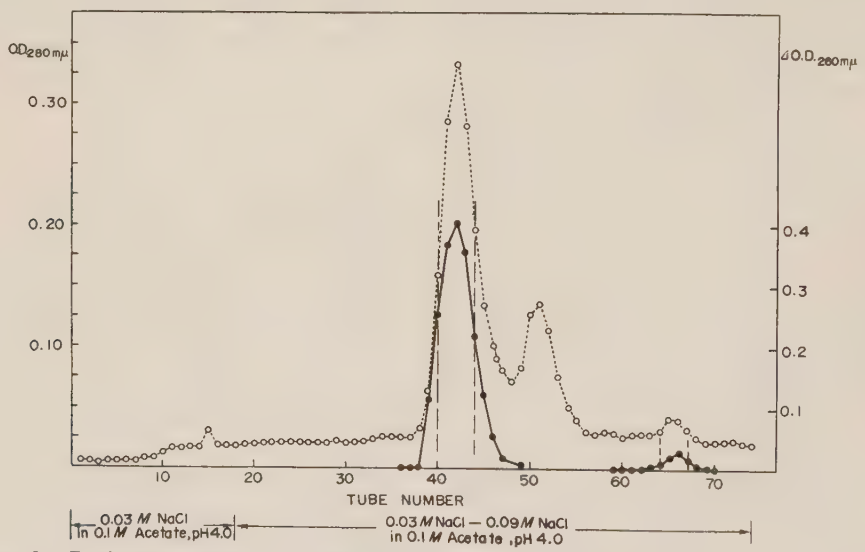

FIG. 2. Rechromatography of RNase of *Streptomyces erythreus*.
—●— RNase activity (Δ. D. at 260 mμ)
--○-- Protein concentration (O. D. at 280 mμ)
Exchanger, 5 g. of DEAE-cellulose; Column size, 14.2 cm. × 3.1 cm.²; Charged material, fraction No. 40—44 of Fig. 1; Fraction volume, *ca.* 9.1 ml./tube. After the material charged, the column was washed with 150 ml. of 0.03 *M* NaCl in 0.1 *M* acetate pH 4.0 (Fraction No. 1—18), then gradient elution was carried out. The mixing flask contained 420 ml. of 0.03 *M* NaCl in 0.1 *M* acetate (pH 4.0) and 500 ml. of 0.09 *M* NaCl in 0.1 *M* acetate (pH 4.0) was charged in reservoir flask. The activity of RNase was determined after 1000-fold dilution with phosphate buffer (0.2 *M* pH 7.4).

column of DEAE-cellulose after washing with 150 ml. of 0.03 *M* NaCl in 0.1 *M* acetate buffer (pH 4.0).

The overall purification was 1,700-fold with a yield of about 6.5 per cent (in fraction No. 40 to No. 44, Fraction A).

The major part of the following studies was carried out on Fraction A, although there was found in the Fig. 2 a very small peak of the RNase activity (Fraction B), to which some references will be given later.

TABLE I

Purification of RNase of Streptomyces erythreus

Step. No.	Activity	Overall Yield	Specific Activity
	u.	%	u.
Starting broth	1,950,000	100	6.5
1. Acrinol precipitate	660,000	34	115
2. Adsorption on DEAE-cellulose	390,000	20	1,200
3. DEAE-cellulose chromatography			
(Frac. A)	127,400	6.5	11,200
(Frac. B)	7,440	0.38	—

Properties of Enzyme Preparations

Stability—The sample of purified RNase preparation obtained as described above (Fraction A) showed remarkable stability. The enzyme solution in 0.1 *M* acetate buffer pH 4.0 could be stored at 2°C for over one month without any decrease in its activity. As shown in Table II, the enzyme preparation showed remarkable heat resistancy. Nearly all of the original activity was recovered after heating at 80°C for 5 minutes at pH 2.0–7.4. Even by heating at 100°C for 10 minutes, the preparation was quite stable at neutral pH, while about 50 per cent of the activity was lost at pH 2.0.

TABLE II

Stability of Streptomyces erythreus RNase

Temperature	Time	pH	Relative activity
	min.		
Control	—	—	100
100	10	7.4	85
100	10	5.6	90
100	10	2.0	51
80	5	7.4	95
80	5	5.6	103
80	5	2.0	85

Fraction A was diluted with buffer (0.1 *M* phosphate, pH 7.4; 0.1 *M* acetate, pH 5.6; 0.1 *M* glycine, pH 2.0). After heating, the samples were diluted 50-fold with 0.2 *M* phosphate (pH 7.4) and activity was determined.

Optimum pH—Influence of pH on the activity of the enzyme was studied with 0.2 *M* phosphate buffer. pH optimum was pH 7.4–7.3.

Specificity—To investigate the specificity of the enzyme, yeast RNA was digested with the preparation (Fraction A) obtained by final DEAE-cellulose column chromatography. Digestion was followed by spectrophotometric method and by paper chromatography. As shown in Fig. 3, rapid increase of O. D. 260 mμ of uranyl reagent soluble fraction was observed in the early stage of the digestion after which period only little increase in 260 mμ absorbancy was observed.

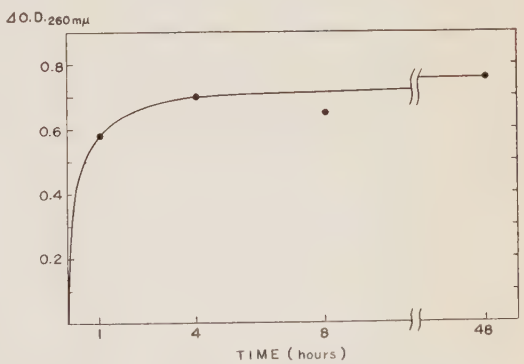

FIG. 3. Digestion of yeast RNA with RNase of *Streptomyces erythreus*.

100 mg. of yeast RNA were dissolved in 4 ml. of 0.2 *M* phosphate (pH 7.4), added 1 ml. of Fraction A (2,800 u.) and incubated at 37°C. 0.15 ml. of aliqout was removed at intervals, diluted with water to 1.0 ml. and 0.2 ml. of uranyl reagent was added. After standing 20 minutes, ppt. was removed, then the 0.25 ml. of supernatant was diluted to 5 ml. with water and the absorbancy at 260 mμ was read.

The fact suggested that the yeast RNA contains some structure resistant to the enzyme action.

The digestion of yeast RNA (10 mg./1.2 ml.) with a small amount of the enzyme (11 u./1.2 ml.) yielded cyclic guanylic acid as a major product at an early stage of the digestion, followed by an appearance of 3'-guanylic acid as revealed by paper chromatography with solvent 1.

The digestion with a large amount of the enzyme (560 u./ml.), as presented in Fig. 3, yielded only 3'-guanylic acid as mononucleotide. These results are illustrated in Fig. 4.

The facts suggested that the enzyme preparation, like RNase T_1, cleaves only the phosphodiester bond between guanylic acid and that 3'-guanylic acid is formed *via* cyclic guanylic acid.

Additional evidence in favour of the above mechanism was obtained with the experiment using cyclic mononucleotides prepared by liquid ammonia degradation of yeast RNA.

As indicated in Fig. 5, only cyclic guanylic acid was digested to 3'-guanylic acid.

The enzyme preparation also attacked pancreas RNase resistant fraction of yeast RNA, but not DNA.

Specificity of RNase in the Minor Peak (Fraction B)—From the results of the following studies, it seems clear that the specificity

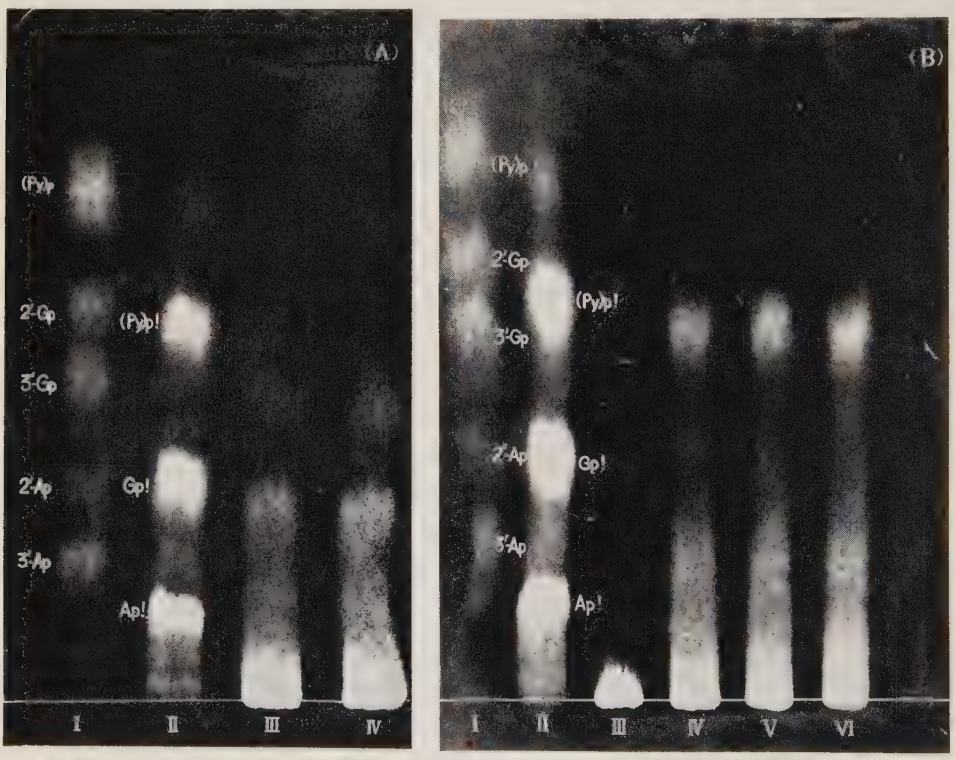

Fig. 4. Paper chromatograms of digests of yeast RNA with RNase of *Streptomyces erythreus*. Solvent 1 was used.

(A). Yeast RNA (10 mg./1.2 ml.) was digested with Fraction A (11 u./1.2 ml.) at 37°C.
(I) Mixture of mononucleotide, (II) mixture of cyclic mononucleotides, (III) digest after incubating for 15 minutes, (IV) digest after incubating for 30 minutes.

(B). The conditions of the digestion were the same with those of Fig. 3.
(I) Mixture of mononucleotides, (II) mixture of cyclic mononucleotides, (III) control (without enzyme), (IV)–(VI) digests after incubating for 1, 4, and 8 hours respectively.

of the RNase found in the minor peak of final chromatography (Fraction B) is apparently the same as that of Fraction A.

Yeast RNA was digested with Fraction B and paper chromatographed with solvent 1. As shown in Fig. 6, spots of 3'-guanylic acid and cyclic guanylic acid were observed besides that of resistant polynucleotides.

Upon digestion of cyclic mononucleotides with this fraction, it was found that only cyclic guanylic acid was hydrolyzed to 3'-guanylic acid (*cf.* Fig. 5).

Base Ratios and Terminal Nucleotides of Resistant Polynucleotides—As found in Fig. 6, both of the enzyme preparations (Fraction A and B) leave a resistant polynucleotide core after exhaustive digestion of yeast RNA. As to these resistant polynucleotides, base ratios were determined and terminal nucleotides of them were tested according to a technique analogous to the one used by Volkin and Chon (*14*).

The digestion mixtures (*cf.* Fig. 6) were dialyzed against distilled water, treated with 0.1 *N* HCl, dialyzed again, and lyophylized.

The base ratios of these samples are shown in Table III.

The resistant nucleotides which were obtained from digestion with Fraction A or B, were treated with 80 u./ml. of prostatic PMase, and then hydrolyzed with *N* NaOH.

Fig. 5. Chromatograms of digests of cyclic nucleotides with RNase preparations of *Streptomyces erythreus*.

The digestions were carried out at 37°C for 2 hours (pH 7.4 in phosphate buffer). Solvent 1 was used.

(I) G_p!, (II) G_p!+Fraction A (28 u./ml.), (III) G_p!+Fraction B (17 u./ml.), (IV) A_p!+$(Py)_p$!, (V) (IV)+Fraction A (28 u./ml.), (VI) (IV)+Fraction B (17 u./ml.).

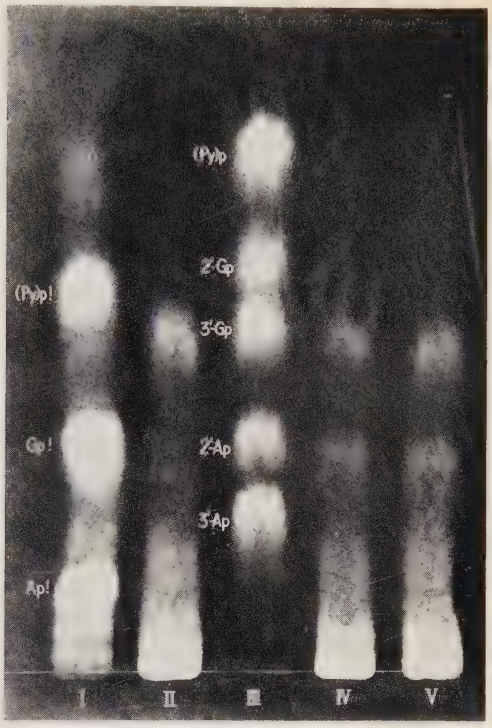

Fig. 6. Chromatograms of the digests of yeast RNA with Fraction A and B.

(I) Mixture of cyclic mononucleotides, (II) digest of yeast RNA (20 mg./ml.) with fraction A (280 u./ml.) at 37°C for 4 hours, (III) mixture of mononucleotides, (IV)–(V) degests of yeast RNA (20 mg./ml.) with Fraction B (83 u./ml.) at 37°C for 4 and 6 hours respectively. Solvent 1 was used.

The nucleosides derived from the terminal nucleotides and mononucleotides arised from internal nucleotides were chromatographically separated on paper using the solvent 2.

Table III

Base Ratios of the Resistant Polynucleotides

	G	A	C	U
Yeast RNA	10.9	10	7.7	9.3
Resistant polynucleotides to Fraction A	6.4	10	8.3	10.2
Resistant polynucleotides to Fraction B	6.8	10	8.3	10

Conditions of the digestions were the same as cited in Fig. 6. Resistant polynucleotides to Fraction B was prepared from 6 hour digested yeast RNA. (*cf.* Fig. 6)

The chromatograms of the hydrolyzates provided from both of the resistant polynucleotides were almost identical. Clear spot of guanosine and very faint spots of nucleosides which were probably derived from original terminal nucleotides were observed.

This suggested that the major part of the terminal nucleotides of resistant polynucleotides is guanylic acid.

Discussion

The results reported above show that a highly active preparation of RNase *Streptomyces erythreus* can be obtained by the combination of the acrinol precipitation and by DEAE-cellulose chromatography. In the early stage of purification, various methods of enzyme precipitation were surveyed. Although it is possible to precipitate the enzyme by fractionation with ammonium sulfate, it was found that the acrinol method described here is preferable in handling the large amounts of starting broth. In case where ammonium sulfate was used, the enzyme precipitated as a soft curd. This caused difficulty in collecting the enzyme. Furthermore, the salt precipitated material could not directly be used for ion exchange chromatography owing to the large quantity of the salt contaminant.

Besides the major peak of the RNase activity (Fraction A), a very small peak (Fraction B, about 1/20 of the total activity of Fraction A) was found in the final chromatographic separation. The results of the studies of these two fractions indicated that both fractions have apparently the same specificity.

This reminiscent of the work of Boman and Kaletta (*15*) in which they obtained three peaks of phosphodiesterase activity from whole venom with chromatography on DEAE-cellulose and suggested that the peaks may represent three protein forms of the same enzyme having arisen by the action of proteolytic enzyme in the snake venom.

During the course of this work it was found that the broth of *Streptomyces erythreus* also contains proteolytic enzymes in a considerable amount. Like the RNase, the protease was thermostable (vetaining 50 per cent of the original activity after heating at 80°C for 5 minutes at pH 2), it co-precipitated with RNase in the acrinol precipitation step, and was eluted slightly later than RNase activity in column chromatogrphy.

Although it seems necessary to study the matter further, there is a possibility that the minor peak of the RNase activity (Fraction B) obtained with final DEAE-cellulose column chromatography might be derived from the same enzyme found in the major component (Fraction A) by action of the proteolytic enzyme.

From the results reported above, it seems clear that the specificities of these enzyme preparations were quite similar to that of RNase T_1 which was found by Sato and Egami in an extract of *Aspergillus oryzae*.

RNase obtained in this experiment split the secondary phosphate ester bond of guanosine-3'-phosphate via guanosine-2',3'-cyclic phosphate as an intermediate. Only 3'-guanylic acid was found as a major

After finshing of this work, the author was informed that RNase with similar specificity was found in the broth of *Streptomyces albogriseolus* by Omura, *et al.* (*16*).

product in the digest of RNA.

It is noteworthy that RNase which has a similar specificity to RNase T_1 was found in the broth of *Streptomyces erythreus* which has taxonomically no relation with *Aspergillus oryzae*.

SUMMARY

1. The RNase of *Streptomyces erythreus* was purified 1,700-fold by the combination of acrinol precipitation and by DEAE-cellulose column chromatography.

2. The enzyme preparation was found to be thermostable.

3. By digestion of yeast RNA with the enzyme preparation, guanosine-2',3'-cyclic phosphate was formed at an early stage, 3'-guanylic acid at a later stage, and other mononucleotide was scarcely detected.

4. Guanosine-2', 3'-cyclic phosphate was the only cyclic mononucleotide which was digested with this RNase preparation.

5. Even after the exhaustive digestion of yeast RNA with this preparation, resistant polynucleotides remained.

6. The terminal nucleotide of the resistant polynucleotides was exclusively guanylic acid.

7. Besides the main peak of RNase activity (Fraction A), a very small peak (Fraction B) was found in DEAE-cellulose chromatography and these two fractions had apparently the same specificity for yeast RNA and cyclic nucleotides.

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A Simple Method for the Fractionation of Globins into their α- and β-Chains

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The fractionation of whole globin into their α- and β-chains has been achieved by several different methods; by elution chromatography (human (*1*), bovine (*2*) and horse (*3*) globins) by continuous electrophoresis (bovine globin (*4*)) or counter current distribution (human globin (*5*)). In addition to these methods, Wilson and Smith (*3*) also reported a partial resolution of horse globin into the valylleucyl and the valylglutamyl chains by fractional precipitation with acidic acetone. Recently the present author found that all the valyl (methionyl in the cases of bovine and sheep globins)-histidyl (β) (*6*) chain of globin (human adult, bovine and sheep) could be precipitated from the 8 *M* urea solution of whole globin with trichloroacetic acid, leaving all the valylleucyl (α) chain in the supernatant.

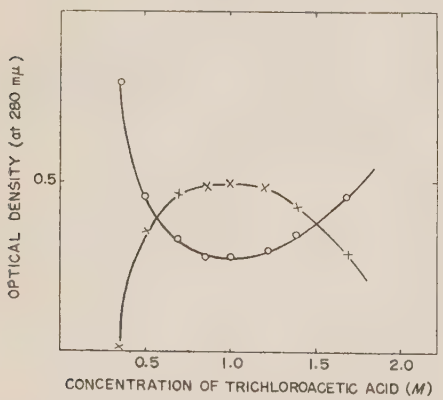

Fig. 1. Effect of the concentration of trichloroacetic acid on the precipitation of globin from 8 *M* urea solution.

—○— Supernatant fraction.

—×— Precipitates fraction (after being washed with the corresponding solvent and redissolved into a definite amount of 8 *M* urea)

Fig. 1 illustrates the relationship between the final concentration of trichloroacetic acid added and the amounts of globin precipitated from the 8 *M* urea solution at a concentration of one mg. per ml. The result indicates that globin in urea solution is only partially and much less easily precipitated with trichloroacetic acid, differing from globin in the absence of urea, which in turn, is completely precipitated with 0.3 *M* trichloroacetic acid.

Fig. 2 is a composite electrophoretic patterns of the supernatant and of the pre-

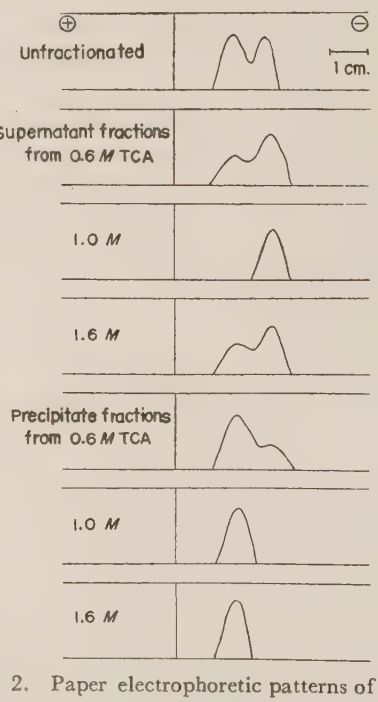

Fig. 2. Paper electrophoretic patterns of supernatant and precipitates derived from whole bovine globin with trichloroacetic acid in 8 *M* urea.

cipitates (after being washed with the corresponding solvent) thus obtained. The faster- and slower-moving components under these conditions, were already elucidated by Take (*4*) to be the bovine globin α and β, respectively, so the result seemed to suggest the quantitative resolution of whole globin into the α (supernatant) and the β (precipitates) with 1 *M* (0.8–1.2 *M*) trichloroacetic acid.

Such a resolution could be also achieved on bovine globin solution of much higher concentration. The procedures are summarized in Scheme I. The both fractions so obtained, were found to be free from other component not only by paper electrophoretic analysis (*4*) but also by N-terminal analysis (*2*). Thus the precipitate- and supernatant-fractions gave mainly DNP-methionine and DNP-valylleucine* respectively on hydrolysis of their DNP-derivatives, and the amount of other ether-soluble DNP-amino acid, if it were present, was less than one per cent.

Using the method shown in Scheme I,

Scheme I

Fractionation of Bovine Globin into the α- and β-Chains

Whole globin 500 mg.

dissolved into 8 *M* urea (30 ml.), added with 2 *M* trichloroacetic acid in 8 *M* urea (30 ml.), stood at room temperature for 5–10 hours

Precipitates	Supernatant and Washings
washed with 1 *M* trichloroacetic acid in 8 *M* urea (twice, 20 ml. each), washed with ether	dialyzed, lyophilized
Globin β	Globin α
280 mg.	175 mg.
N: 16.92 per cent	N: 16.23 per cent

human and sheep globins were also resolved into their α- and β-chains** with success, while hen, snake, pig and horse globins were not (analyzed both by electrophoresis and by DNP-method). According to Satake,*** the former two globins contained only a pair of valylleucyl chain, so no resolution seemed not to be surprising. In the cases of the latter two, resolution was not quantitative. These globins possessed valylglutamylleucyl chain (*3*) which was absent in human, bovine, sheep, hen and snake globins (*9*) and also possessed valylleucyl chain which was reported to have slower (not faster, compare with Fig. 2) electrophoretic mobility (*3*). Thus the optimum condition for the resolution might be different in these cases.

Previously, Satake (*7*) and the present author (*8*) fractionated oxidized insulin A and B chains (from beef, whale and bonito fish) by fractional precipitation of the latter with trichloroacetic acid (*7*) and with perchloric acid (*8*), without accompanying any hydrolysis of peptide linkages. The facts seemed to suggest that there occurred scarcely such an undesirable hydrolysis in the present case, too. The fractionation of globins with trichloroacetic acid in the presence of 8 *M* urea, either in analytical or preparative scale, seems to be far convenient as compared with any of the methods already reported, and to be usefull for the analysis of abnormal chain (either α or β) in human hemoglobin mutants.

* Acid hydrolysis at 110°C for only a half hour (*5*).

** DNP-valine but no DNP-valylleucine was obtained from human globin β (see (*5*)).

*** Unpublished (Satake, K) (see also (*9*, *10*)).

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The Presence of Two Lactic Dehydrogenases in *Piricularia oryzae*

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There have been many reports on the enzymes catalyzing the oxidation of lactate to pyruvate from different sources and on the specificities of these enzymes for the stereoisomeric substrates and the hydrogen acceptors (*1–5*). The authors investigated the lactate oxidation by the enzyme obtained from *Piricularia oryzae* (rice blast disease fungus), and found two distinct systems catalyzing the dehydrogenation of lactate to pyruvate; one is active specifically on D-isomer (D-LDH*) and the other on L-isomer (L-LDH). Experimental results are briefly described below.

P. oryzae (No. 9–5828) was grown on standard media (*6*). Harvested mycelia were ground with sea sand and extracted with twice their weight of 0.1 *M* phosphate buffer (pH 6.3). The sand was then centrifuged off. Ammonium sulfate was added to the supernatant to make an 80% saturation. The resulting precipitate was collected and redissolved in the same buffer solution. To this solution was added a 0.05 volume of 1% rivanol solution. The precipitate formed was discarded and ammonium sulfate was added to the supernatant to make an 80% saturation. The precipitate was again dissolved in water and ammonium sulfate again was added to make another 80% saturation. Such repeated precipitations with ammonium sulfate led to the elimination of endogenous substrates and of cofactors. The precipitate was then dissolved in 0.1 *M* phosphate buffer of pH 6.3. This solution was used as the enzyme preparation.

The substrate and coenzyme specificities of the lactate dehydrogenation are shown in Table I. As shown in this Table, the enzyme preparation catalyzes the reversible dehydrogenation of D-lactate to pyruvate in the presence of DPN as a hydrogen acceptor. L-Lactate was also dehydrogenated by the same preparation even in the absence of pyridine coenzyme. In the latter case, 2, 6-DPIP was found to be utilized as a hydrogen acceptor but methylene blue was not*. Quantitative

Table I

Substrate and Coenzyme Specificities of Lactate Dehydrogenation by Enzyme Preparation from P. oryzae

No.	Substrate	Hydrogen carrier	Change in absorbancy	Wavelength employed for measurement (mμ)
1	L-Lactate	2, 6-DPIP	−0.170	600
2	L-Lactate	DPN	0.000	340
3	D-Lactate	2, 6-DPIP	0.000	600
4	D-Lactate	DPN	0.039	340
5	Pyruvate	DPNH	−0.103	340

Incubation mixture contained followings: 50 μmoles of substrate (neutralized with NaOH), 1 μmole of indicated coenzyme or 0.5 ml. of 0.001% 2, 6-DPIP, 0.5 ml. of enzyme solution. Final volume was made up to 3.0 ml. by adding 0.1 *M* phosphate buffer (pH 7.3). Measurement was made by Beckman spectrophotometer model DU at 30°C. Change in absorbancy was measured during period from 15 to 195 seconds after initiation of reaction.

* Abbreviations: LDH, lactic dehydrogenase; 2, 6-DPIP, 2,6-dichlorophenol indophenol; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide.

* The natural hydrogen acceptor remains unclarified.

formation of pyruvate from L-lactate was confirmed (disappearance of L-lactate, 175 μ-moles; formation of pyruvate, 152 μmoles). By adding ammonium sulfate to make a 50% saturation, D-LDH was precipitated, whereas L-LDH remained in the solution. D-LDH was purified 16-fold by ammonium sulfate fractionation being freed from L-LDH. Further purification of L-LDH was unsuccessful. By heating at 40°, 50°, and 60°C for 3 minutes, L-LDH lost its activity by 55, 83, and 85% respectively, whereas D-LDH lost 0, 9, and 50% respectively. pH optima of the two LDH's were 8, though the shapes of their pH curves differed from each other considerably.

No activity of lactic racemase was detectable in the extract of *P. oryzae*.

The authors express their thanks to Dr. H. Katagiri of Kyoto University for his kind supply of D- and L-lactate.

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Effect of Some Drugs on Lactonase Activity of Rat Liver

It has been reported that various drugs, such as Chloretone, barbital, antipyrine, 3-methylcholanthrene, and 3,4-benzpyrene, stimulated the synthesis of L-ascorbic acid from glucose through glucuronic acid pathway (*1*). Other studies showed that such drugs increased the activity of microsomal enzymes, such as azo dye demethylase, *etc.* (*2*). Furthermore, it was reported (*2*, *3*) that ethionine prevented the enhancement of ascorbic acid excretion by drugs as well as the formation of azo dye demethylase induced by 3-methylcholanthrene, and that methionine completely nullified the ethionine block. However, the mechanism for increase of L-ascorbic acid excretion by drugs has not been clarified as yet.

In the present paper is reported the effect of drugs on the lactonase activity which seems to concern L-ascorbic acid metabolism.

Rats were kept in individual cages. Oxalic acid was used to prevent ascorbic acid degradation in 24 hour urine. Rats were fasted but permitted free access to water for 48 hours and were then given evaporated milk (Carnation Milk) in sufficient quantity for seven days. Ascorbic acid in daily urine decreased to almost the constant level of one tenth of the normal excretion. After seven days the rats were injected with 25 mg. per day of sodium 5,5-diethyl-barbiturate or 25 mg. per day of antipyrine, or were given orally 20 mg. per day of Chloretone for several days. During this period evaporated milk was also given. The liver from freshly killed rats was homogenized in 4 volumes of cold 0.25 *M* sucrose. The resulting homogenate was centrifuged at $9,000 \times g$ for 10 minutes and the residue was discarded. The microsomes, isolated from the supernatant by centrifugation at $54,000 \times g$ for 60 minutes, were resuspended in 1 ml. of 0.25 *M* sucrose per g. of wet liver. Enzyme activity was assayed by the manometric method described in Yamada's paper (*4*). The protein content of the solution was assayed by the method of Warburg and Christian (*5*). L-Ascorbic acid was measured by the method of 2,6-dichlorophenol indophenol.

The present experimental results agreed with those of the previous report (*1*) that the ascorbic acid excretion was greatly accelerated by sodium 5,5-diethylbarbiturate, antipyrine and Chloretone. As shown in Fig. 1 the effect of Chloretone on excretion of L-ascorbic acid was more rapid and greater than that of antipyrine and sodium 5,5-diethylbarbiturate. It was found that the liver weight

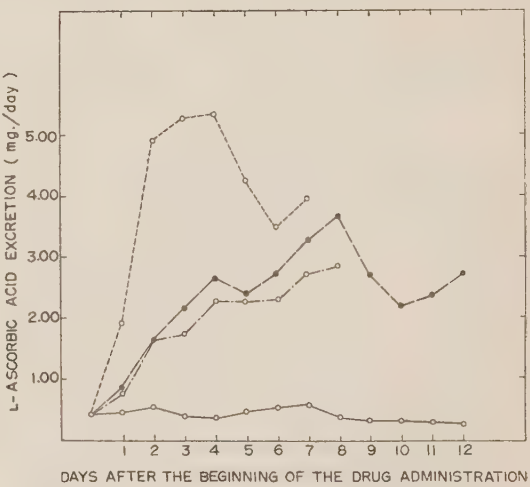

Fig. 1. L-Ascorbic acid excretion.
—○— Control
--●-- Sodium 5,5-diethylbarbiturate
-·-○-·- Antipyrine
---○--- Chloretone

of a rat treated with the drugs was higher than that of a non-treated rat. For the assay of lactonase I (soluble), the activity in the supernatant obtained through the isolation of microsomes was determined with D-gulonolactone. For the assay of lactonase II (microsomal), the activity of the microsomes on D-glucuronolactone was determined. The con-

TABLE I

The Activity of Lactonase II

Experimental group	Duration of drug-treatment (days)	Activity of lactonase II							
		per g. of wet liver				per liver			
		C.[1]	Bar.	Ant.	Chl.	C.	Bar.	Ant.	Chl.
I	8	34	187	72		206	1590	564	
		48	58	79		226	485	520	
		75	87	60		375	660	624	
II	7	96	191		152	527	1570		1060
III	4	153	182		86	865	1490		
			216				1620		
IV	8	102	145			645	1450		
		82	189			650	2080		
		121				835			
V	3	192		263	168	1730		1990	1680
		173		232	200	1560		1740	1860
VI	5	260	275			1870	3020		
		223				1940			
VII	12	115	208			1020	2040		
			216				1770		
			113				1340		
			203				2070		

1) C.: Control; Bar.: Sodium 5,5-diethylbarbiturate; Ant.: Antipyrine; Chl.: Chloretone.

The reaction mixture contained 0.4 ml. of 0.1 *M* sodium bicarbonate, 0.1 ml. of 0.1 *M* magnesium sulfate, 0.3 ml. of 0.001 *M* glutathione, and microsomes obtained from 1 g. of wet liver, and 0.3 ml. of 0.1 *M* D-glucuronolactone was added from the side arm. Total volume was made to 3 ml. Gas phase, 93 per cent of N_2 and 7 per cent of CO_2; temperature, 37°C; pH 7.2.

Activity is expressed as microliter of CO_2 evolved during initial 5 minutes.

ditions are shown in Table I. The lactonase I activity per mg. protein, per g. of wet liver, or per liver showed no difference between drug-treated and non-treated rat liver. However, it was found, as shown in Table I, that the lactonase II activity of drug-treated rat liver per g. of wet liver was higher than that of non-treated rat liver. As the weight of the drug-treated rat liver was higher, the activity of lactonase II per such a liver was markedly higher than that of the non-treated rat liver. Both the excretion of L-ascorbic acid and the lactonase II activity reached a a maximum in 7–8 days after the beginning of sodium 5,5-diethylbarbiturate administration.

Assuming that lactonase II is concerned with the L-ascorbic acid biosynthesis, the increase of the L-ascorbic acid excretion may be due to the increase of the lactonase II activity.

The authors wish to express their gratitude to Prof. N. Shimazono for his kind guidance and continuous encouragement.

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Does EDTA* Bind to Actomyosin?

Based on various evidences (*1–5*), S. Ebashi has presented a view (*1–3*) that the relaxing action of EDTA on muscle models is due to the removal of essential calcium from actomyosin. Parker and Gergely (*6*), however, opposed this view, stating that EDTA might act on actomyosin-system by its direct binding to actomyosin. Bozler** and S. Ebashi (*3*) could not find this binding, but their procedures used for measuring EDTA were not sensitive enough to detect a minute amount of the binding; therefore, their experiments might not appear to be a definite counterevidence against Parker's view.

TABLE I

Binding of EDTA to Actomyosin

Radioactivity per ml.	A	B	C
	c.p.m.	c.p.m.	c.p.m.
Supernatant	5,679	7,522	7,495
Residue	5,583	7,372	7,274
Calculated Activity in Residue	5,580	7,400	7,370

Ten ml. solutions, composed of 0.1 *M* KCl, 0.02 *M* Tris-maleate buffer (pH 6.8), 42 mg. actomyosin and specified concentrations of EDTA, *viz.*, 0.1 m*M* in A (containing C^{14}-EDTA, 60 μc per mmole EDTA), or 1 m*M* in B and C (containing C^{14}-EDTA, 8 μc per mmole EDTA; C contained 1 m*M* $MgCl_2$ in addition to the above-mentioned), were centrifuged at 4,000×*g* for 10 minutes and their supernatants were taken off. The residues were carefully weighed: A, 1.73 g., B, 1.91 g. and C, 1.88 g. Then the radioactivities of supernatants and residues were measured by routine procedures respectively. Figures in table show the number of counts per minute per ml. of samples corrected to infinite thinness. Experimental errors of these values were within 2 per cent. Calculated values were obtained under the assumption that EDTA did not combine with actomyosin but was distributed only in the space of the medium around actomyosin molecules in the same concentration as that of the supernatant. In all calculations, 0.73 was used as the specific volume of actomyosin.

In the present communication the binding between EDTA and actomyosin was determined by the use of (carboxy-C^{14})-EDTA***. As a result the author also failed to find any of the binding in agreement with the experiments mentioned above. Even though we take some experimental error into account, bound EDTA to actomyosin should be less than 0.02 mole per 10^5 g. actomyosin.

These observations not only difinitely substantiate the S. Ebashi's opinion as to the mechanism of the relaxing action of EDTA, but also lend a strong support to his concept (*1–3*) that the calcium accumulating activity of the natural relaxing factor accounts for the physiological action of the factor.

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* Following abbreviations were used: EDTA, ethylenediaminetetraacetic acid, and Tris, tris (hydroxymethyl) aminomethane.

** Personal communication.

*** Obtained from Daiichi Pure Chem., Co., Tokyo.

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Removal of Bound Nucleotide and Calcium of G-Actin by Treatment with Ethylenediaminetetraacetic Acid*,**

The mechanism of the G-F transformation of actin has been investigated by many workers. Straub and Feuer (*1*) demonstrated that ATP bound to G-actin is hydrolyzed to ADP, accompanied by the G-F transformation. Kuschinsky and Turba (*2*) reported that the transformation is inhibited by SH reagents. Feuer *et al.* (*3*) and Hasselbach (*4*) emphasized the role of the divalent metal in the transformation. The work that is reported below was performed with a view to get information as to the reaction mechanism of divalent metal in the transformation.

G-actin was extracted from acetone dried rabbit muscle powder and purified by the ultracentrifugal technique of Mommaerts (*5*). G-actin was transformed to the F-form by the addition of 0.1 *M* KCl at 20°C and pH 8.3, unless otherwise stated. The course of the transformation was followed by measuring the increase in the reduced viscosity.

The G-F transformation of actin was inhibited completely by the addition of chelating compounds, such as pyrophosphate, 1, 2-cyclohexanediaminetetraacetic acid and especially by EDTA. As shown in Fig. 1, the concentration of EDTA necessary to stop the polymerization of G-actin was independent of whether EDTA was added at the stage of G-actin or after the polymerization occurred partially. When the incubation time of G-actin with 5 m*M* EDTA in the presence of 0.2 m*M* ATP and 0.1 *M* KCl was long, G-actin was not transformed to the F-form even by the addition of excess (6 m*M*) Mg^{++}: for 1 and 3 hours' incubation with EDTA the percent losses of the ability of the transformation were 60 and 100, respectively. Furthermore, when G-actin was dialyzed against ATP after the treatment with 5 m*M* EDTA in the presence of 0.2 m*M* ATP and at 20°C and pH 8.3, it did not polymerize on the addition of 0.1 *M* KCl, Mg^{++} and/or Ca^{++}.

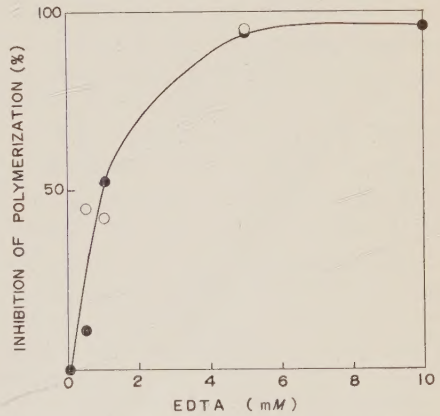

FIG. 1. Inhibition by EDTA of polymerization of G-actin. —●—, EDTA was added to G-actin. —○—, EDTA was added after 50% of G-actin was transformed to F-actin. The reduced viscosity was measured 8–10 hours after addition of EDTA. 0.86 mg. of actin/ml. 0.1 *M* KCl, pH 8.3, 20°C.

Table I shows the contents of the bound nucleotide and calcium in G-actin expressed as moles per mole of actin monomer (molecular weight, 6.1×10^4 g. (*6*)), before and after the treatment with EDTA. The content of calcium was determined by Yanagisawa's method (*7*) with slight modifications (*8*) after the removal of EDTA. To measure the bound nucleotide, free ATP was removed from G-actin preparation by passing G-actin through a column of Dowex 1-X2 (Cl^- form,

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** The following abbreviations will be used: ATP adenosine triphosphate, ADP adenosine diphosphate, EDTA ethylenediaminetetraacetic acid.

pH 8.2-8.6) (*9*). After the deprotenization by the addition of 6% perchloric acid, the optical density at 259 mμ of the filtrate was measured. As clearly shown in Table I, both the

TABLE I

Contents of bound Ca^{++} and Nucleotide of G-actin[1)]

	Control actin	EDTA treated actin
Ca^{++}	1.52	<0.15
Nucleotide	1.1	0

1) Expressed as moles/mole of actin monomer.

nucleotide and Ca^{++} bound tightly to G-actin were completely removed by the EDTA treatment. In 0.6 *M* KCl the G-actin treated with EDTA did not bind to myosin A, and in 0.075 *M* KCl and 2 m*M* Mg^{++} ATPase activity of myosin A was not activated by the addition of the EDTA treated G-actin.

It is well known that the G-F transformation is accompanied by the dephosphorylation of bound ATP (*1*) and that G-actin is denatured irreversibly after the removal of bound ATP (*1*, *9*). Therefore it is reasonable to conclude that ATP is bound to Ca^{++} in G-actin and that, by the removal of Ca^{++} by EDTA, ATP is separated from G-actin and then G-actin is denatured irreversibly.

After this work had been completed, Dr. L. Noda of Dartmouth Medical School informed us by personal communication that M. Barany, R.C. Strohman and A. Martonosi presented results similar to ours at the Federation Meeting of American Societies for Experimental Biology, at Atlantic City in April 1961.

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